Characterisation of the IgE Response in *Nippostrongylus brasiliensis* infected rats

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DECLARATION

I hereby declare that:-

(i) this thesis has been composed by myself

(ii) it has not been accepted in any previous application and,

(iii) the work described here was conducted by myself except when acknowledged otherwise.

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ABSTRACT OF THESIS (Regulation 3.5.10)

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Allergens from somatic extracts of adult *Nippostrongylus brasilinesis* worms were characterized by SDS-PAGE and Western blotting. Seven allergens, with molecular weight ranging from 14,000 - 69,000 were identified after blotting with hyperimmune serum (HIS) from rat immunized by several infections with *N. brasiliensis*. Monoclonal mouse anti rat-IgE was used a a specific probe. The major 14,000 - 17,000 MW allergens, partially purified by electrolution, retained activity by passive cutaneous anaphylaxis (PCA). Other immunoblobulin isotypes, notably IgG_1, also bound to allergens on Western blot. The specificites of other immunoglobulin isotypes and IgE were compared by sequential incubation of blotted allergens with IgE-depleted HIS and with affinity-purified serum IgE. Despite retaining biological activity by PCA, the affinity-purified IgE proved to be insufficiently specific for use on Western blots. The range of allergens detected and the kinetics of appearance of parasite-specific IgE differed between LOU, Hooded Lister, August and F334 undergoing primary infection with *N. brasiliensis*. Whereas August rats responded uniformly, there were variations in the timing and specificity of IgE responses between individual rats within the other strains. Nevertheless, it was possible to classify LOU and Wister rats as early and F334 rats as late responders. Internalization of IgE within the cytoplasm fo intestinal mucosal mast cells (MMC) of rodents infected with intestinal nematodes, and the absence of this isotype from the cytoplasm of connective tissue mast cells (CTMC) suggested that the MMC granule protease, rat mast cell protease II (RMCP II), might fail to catabolize IgE. However, when compared with the granule protease RMCP I from CTMC, RMCP II was more efficient in catabolizing the heavy chains of both IgE and IgG_{2a}. The light chains wee apparently resistant to digestion. The presence of IgE-bearing cell populations in bone marrow, peripheral blood, and peritoneal cavity, was monitored by flow cytometry in normal Wister rats and during the course of infection with *N. brasiliensis*. The proportions of IgE-bearing cells in the peritoneum and bone marrow increased on day 10 and, in peripheral blood on day 15. The IgE-bearing cells in each compartment were of mixed phenotype.

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CONTENTS

ABBREVIATIONS

CFA	Complete Freund's Adjuvant
DNP	Dinitrophenyl
EB	Evan's Blue
ES	Excretory/Secretory antigens
$Fc_\varepsilon R$	Receptor for IgE
$Fc_\varepsilon RI$	Receptor I for IgE Fc
$Fc_\varepsilon RII$	Receptor II for IgE Fc
HIS	Hyperimmune sera
Ig	Immunoglobulin
LT	Leukotriene
L	Larval/adult stage
MHC	Major Histocompatibility Complex
MMC	Mucosal Mast Cells
MLN	Mesenteric Lymph Node
MW	Molecular Weight
mg	Milligram
nK/mg	Nanokatal/milligram
NaDoc	Sodium Deoxychollate
NRS	Normal Rat Serum
PAF	Platlet Activating Factor
PBL	Peripheral Blood Leukocytes
PCA	Passive Cutaneous Anaphylaxis
pI	Isoelectric point
PK	Prausnitz Kutzner test
RAST	Radioallergosorbent Test
RBL	Rat Basophil Leukaemia Cell Line
RIA	Radioimmunoassay
RIST	Radioimmunosorbent Test
RMCP	Rat Mast Cell Protease
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
T cells	Thymic lymphoid cells
μg	micrograms
B cells	Bursa lymphoid cells.

CHAPTER 1

GENERAL INTRODUCTION

INTRODUCTION

Parasitic nematode infections have been a major problem to both man and his domestic animals for many thousands of years. Nematodiasis in man is particularly common in the tropics where poor sanitation, malnutrition, and inadequate medical facilities predominate. Furthermore, the tropical climate favours parasite survival. Nematodiasis in domestic animals has a much wider geographic distribution, including many temperate regions.

Accurate epidemiological studies are largely unavailable, but a recent report, conducted in Africa, estimated that 32% of Africa's 480 million inhabitants harbour the intestinal nematode *Ascaris lumbricoides* (Crompton and Tulley, 1987). Many individuals were concomitantly infected with at least one other parasitic nematode. The morbidity and fatalities caused by nematodes as a whole are almost impossible to quantify. *A. lumbricoides* alone has been estimated to cause 20,000 deaths/annum and hookworm 55,000 deaths/annum on a global scale (Pritchard, 1986).

Host-nematode relationships have been extensively studied over the last 25 years and particular emphasis has been placed on the host's immune effector mechanisms. Our knowledge of crucial host-parasite interactions which determine the nature and efficacy of anti-parasite effector mechanisms is still limited. Some parasitic nematode infections are terminated within relatively

short periods but many parasites survive for months or even years. Filarial nematode infections in man and animals frequently proceed to chronicity and can persist for the life-time of the host (reviewed in Selkirk, Denham, Partono, Sutano and Maizels, 1986). *A. lumbricoides* is capable of chronic debilitating infection and has been reputed to survive in the host's intestine for as long as twenty years (Pritchard, 1986).

Parasitic and free-living nematodes share a number of structural and physiological characteristics. All are enclosed in a tough cuticle composed of collagens and other structural proteins (McBride and Harrington, 1967 ; Leushner, Semple and Pasternak, 1979 ; Cox, Kursch, and Edgar, 1981). The cuticle was formerly thought to be inert, functioning solely to contain and protect the parasite (Lumsden, 1975). However, this view has been modified. The cuticle is probably involved in important physiological functions such as gas-exchange, maintenance of homeostasis (MacKenzie, Preston and Ogilvie, 1978) and absorption of nutrients (Howells and Chen, 1981).

Parasitic nematodes, characteristically, have five developmental stages, each of which is separated by moulting of the cuticle (reviewed by Lumsden, 1975 and Johnson, Grundy and Van Thompson, 1970). The cuticle ranges from 0.15 to 0.5 microns in thickness (Leushner *et al.*, 1979) and is subdivided into the outer cortex, the medulla and the basal fibre-containing layer. It is an extremely resilient structure and may have immunosuppressive properties (Archer, Coulis, Jindra and Robson, 1985). Nematodes

possess a simple but functional digestive tract, the pharynx and rectum having a cuticular covering continuous with the exterior. The intestine has a fine basement membrane which supports a single layer of cells (Kassai, 1982) involved in adsorption and production of degradative enzymes (Gentner and Castro, 1974; Fujino and Ishi, 1986).

The Life-cycle and Biology of *Nippostrongylus brasiliensis*

The allergens of the nematode parasite *N. brasiliensis* and the IgE responses of the rat, the natural host of this intestinal nematode, are the principal topics of this thesis. It is therefore appropriate, at this stage, to introduce the life-cycle of *N. brasiliensis* which primarily naturally infects wild rats and, less frequently, wild mice. The life-cycle is similar in many respects to that of other parasitic nematodes. Lumen-dwelling adult worms produce large numbers of eggs which are passed in the faeces. The eggs hatch in the soil to produce first stage larvae, L_1, which mature through second, L_2, and third, L_3 stages under appropriate conditions of moisture and warmth (> 27^{o}C). On contact with host skin, the infective L_3 penetrates and migrates to the lungs where it moults 2-3 days after infection to become L_4. The L_4 passes via the upper airways to the pharynx where it is swallowed and moults to become the L_5 in the small intestine where it matures further. The worms assume their natural predilection

site in close proximity to the surface of the intestinal mucosa (Alphey, 1970) and commence egg laying on days 5-6. They are expelled from the gut, approximately 12 to 14 days after infection (Sarles and Taliaferro, 1936).

Nematode Antigens

Attempts have been made to characterize nematode antigens and allergens of potential importance in immunity. Nematode antigens as a whole have been conveniently grouped into three categories: (1) somatic and structural proteins; (2) cuticular antigens and (3) excreted and secreted (ES) antigens. During infection, the host is primarily exposed to antigens in categories 2 and 3 and only later to somatic antigens when, and if, the integrity of the parasite has been disrupted (Boyce, Branstetter and Kazacos 1988; Kennedy, Tiernay, Ye, McMonagle, McIntosh, McLaughlin and Smith 1988; Aguila, Cuella and Guillen, 1988). Immune responses to antigens from ES and cuticle are thought to be largely responsible for the induction of host protective responses (Ortega-Pierres, MacKenzie and Parkhouse, 1984). Allergens are a major component of ES preparations and may also be relevant to immunity. Somatic antigens are probably of little significance to the generation of effective immune responses (Kobayashi, Kuemada and Ishizaka, 1972).

Techniques such as maintenance of parasites *in vitro*, labelling of surface antigens and detergent stripping of epicuticle were specifically designed to define potentially protective cuticular

and ES antigens. These methodologies have been used extensively in the characterization of intestinal and filarial nematode antigens. The techniques are complemented by the use of radiolabelling methods which increase both the sensitivity of detection and the identification of the source of antigen.

Five reagents for radio-iodination which include Chloramine-T, Bolton-Hunter reagent, Lactoperoxidase, Iodosulphanilic acid and Iodogen have been used to radio-label protein components of the nematode surface (Marshall and Howells, 1985; reviewed in Philipp and Rumjaneck, 1984; Baschong and Rudin, 1982).

The relative labelling efficiencies of these reagents have been compared by electron microscopy (Baschong and Rudin, 1982; Marshall and Howells, 1985) and advantages and disadvantages were attributed to each reagent. For example, chloroglycoluril (Iodogen), the iodination catalyst, is relatively insoluble in aqueous solution and this may reduce its efficiency under certain circumstances.

Sodium deoxycholate (NaDoc), an anionic detergent, and Cetyltrimethyl ammonium bromide (CTAB) have been employed to remove selectively epicuticle proteins from certain parasites (Pritchard, Crawford, Duce and Behnke, 1985). CTAB was the most efficient, yielding larger quantities of epicuticle proteins from

Nematospiroides dubius and without inducing demonstrable damage to the cuticle (Pritchard *et al.*, 1985). Our own preliminary studies showed that CTAB kills adult *N. brasiliensis* within an hour of treatment.

Developmental Stage-specific restriction of Nematode Surface Antigen Expression

Extraction of radioiodinated surface antigens, using detergents, has demonstrated that some parasites express stage-specific epicuticle proteins. This has been shown for *N. brasiliensis* (Maizels *et al.*, 1983), *Necator americanus* (Pritchard, Behnke, Carr and Wells 1986), *Trichuris muris* (Preston, Jenkins and McLaren, 1986) and *N. dubius* (Pritchard, Maizels, Behnke and Appleby, 1984). Restriction of surface antigen expression may be a physiological necessity or may help the parasite to evade the immune response in some way. *Trichinella spiralis* surface antigens are the most intensively studied and best characterized of this group (Philipps, Parkhouse and Ogilivie, 1980; Almond and Parkhouse, 1986; Ortega-Pierres, Clark and Parkhouse 1986; Silberstein and Despommier, 1984). Besides the stage-specific restriction of *T. spiralis* proteins, it was also apparent that a restricted number of antigens were detected on the parasite surface. Three proteins were detected on the surface of infective and newborn larvae and four on the surface of the intestinal stage (Philipp *et al.*, 1980). However, these results depend upon the sensitivity of the detection methods and it is likely that other proteins are present in low concentrations.

Characterization of the Surface Antigens from *Nippostrongylus brasiliensis*

The surface antigens of the skin-penetrating and pulmonary L_3, and of the enteric L_5 stages of *N. brasiliensis* were characterized by Maizels *et al.* (1983). Infective and pulmonary *N. brasiliensis* L3 expressed surface proteins with molecular weights of 65,000, 41,000, 28,000 and 16,000, and immunological studies indicated that these proteins were antigenically distinct. When compared with *T. spiralis*, *N. brasiliensis* expressed a less-restricted surface antigen profile with seven radio-labelled antigens being identified on pulmonary L_3 and on adult L_5 recovered from the intestines of infected rats (Maizels *et al.*, 1983).

Adult male *N. brasiliensis* express a 90,000 MW surface protein which is apparently lacking from the female and may represent a male-sex associated structure on the worm surface. (Maizels *et al.*, 1983). Sex-associated protein restriction has been identified in two other nematode species, *T. spiralis* and *Brugia pahangi* (Philipp *et al.*, 1980; Ortega-Pierres, Cheyen, Clark and Parkhouse, 1984; Sutanto Maizels and Denham, 1985) and may be a more widespread characteristic.

Our understanding of the biochemistry of the nematode cuticle is still limited. For example surface antigens from *T. spiralis* were extracted and separated by SDS-PAGE and many were subsequently shown to be glycoproteins (Ortega-Pierres *et al.*, 1984). Similarly

the predominant surface protein(MW 60,000), isolated from adult *T. spiralis*, is heavily glycosylated (Ortega-Pierres *et al.*, 1986). However, Ortega-Pierres *et al.*, (1986) reported only poor surface staining with lectin-FITC. Poor surface staining was also observed when adults were incubated with anti-carbohydrate monoclonal antibodies (Ortega-Pierres *et al.*, 1984). Cuticular glycoproteins may be restrictively orientated to minimize exposure of glycoproteins on the parasite surface (Ortega-Pierres *et al.*, 1984). Further restriction was observed with FITC-labelled lipids inserted into the nematode epicuticle. Lateral diffusion of lipids, a normal process in unit membranes, was not observed on the nematode cuticle when a small area of the surface was bleached by laser (Kennedy, Foley, Kuo, Kusel and Garland, 1987). The reason for this restriction has yet to be defined. However, it may reflect another adaptation process that facilitates avoidance of the immune response.

Excreted and Secreted Antigens of Nematodes

Parasitic nematodes, in common with most helminths, secrete and excrete products during infection. The stichosome, in some species, and excretory canals and gut are the main sources of released products but, because of the problem of sampling these secretions in the host, they are best obtained from *in vitro* nematode culture (Kobayashi *et al.*, 1972; Hotez, Trang, McKerrow and Cerami, 1985; Dresden, Rege and Murrell, 1985; Knox and

Kennedy, 1988). Parasite products collected from these cultures are believed to be representative of those released *in vivo*, but even so, some workers still refer to the antigens as *in vitro*-released products.

Unfortunately, the exact nutrients required for prolonged *in vitro* maintenance of nematode parasites are still poorly defined although there are some exceptions. Maizels *et al.* (1984) sustained cultures of larval stages of *Toxocara canis* for two or more months at a time. Generally for most nematodes only limited success has been reported with adult worm cultures (Burt and Ogilvie, 1975). Short-term cultures provide very limited quantities of ES products, nevertheless adult ES antigens have been characterized for a number of nematode species.

The ES products comprise a heterogeneous collection of proteins and glycoproteins, with wide-ranging molecular weights (Brindley, Gam, Pearce, Poindexter and Neva, 1988). Allergens and enzymes are common components of ES products but their role in parasite maintenance has yet to be defined.

Maizels, De Savigny and Ogilvie (1984) and Philipp *et al.* (1980) noted contamination of *T. canis* and *T. spiralis* parasite ES preparations with surface components. Shedding of epicuticular antigens can be demonstrated by loss of radio-label from the surface of the parasite. Surface antigen shedding has been described for *N. brasiliensis* (Maizels *et al.*, 1983), *Strongyloides stercoralis* (Brindley *et al.*, 1988) and *N.*

americanus (Pritchard *et al.*, 1986. This phenomenon may be commonplace among parasitic nematode species and could reflect true *in vivo* events. Surface antigens are also released in large quantities during moulting, (Kobayashi *et al.*, 1972) but this is not always the case since some parasites may resorb the old cuticle (Johnson *et al.*, 1970).

Adult *T. spiralis* produce live larvae and this distinguishes them from other enteric parasitic nematodes. *T. spiralis* larvae invade the intestinal mucosa and migrate via the blood stream or lymphatic vessels to skeletal muscle (Gardiner, 1976) where they develop within protective capsules capable of resisting assault from the host's immune response. In this environment, they produce a number of ES products which adhere to the cuticular surface (Pritchard, 1986). Two such proteins, of 48,000 and 50/55,000 MW were affinity-purified and, when used in vaccination studies, partially protected susceptible mice against a potentially lethal infection with *T. spiralis* (Silberstein and Despommier, 1984). Vaccinated animals were more resistant to migrating muscle larvae than controls, but as would be expected with a stage-specific vaccine, there were no observable effects on the intestinal adult worm burden (Silberstein and Despommier, 1984).

Enzymes in ES Preparations

A range of enzymes has been identified in nematode ES preparations (reviewed by Maizels and Selkirk, 1988). Acetylcholinesterase is secreted by many parasites (Sanderson and

Ogilvie, 1971; Ogilvie and Rothwell, 1973; Rothwell, Ogilvie and Love, 1973), superoxide dismutase, has been identified in adult *T. spiralis* secretions, (Rhodes, 1983) and proteases are common ES components. Proteases have been characterized in larval secretions of *A. suum* (Knox and Kennedy, 1988), *Strongyloides ransomi* (Dresden *et al.*, 1985) and from adult *Ancylostoma caninum* secretions (Hotez and Carami, 1983). In the latter instance, the protease is thought to aid tissue penetration, and because of its anti-coagulant properties, enhance feeding and survival of the adult worm (Hotez *et al.*, 1985). Matthews (1982) and later Kennedy, Tierna, Ye, McMonagle, McIntosh, McLaughlin and Smith, (1988) identified a protease in *Anisakis caninum* larval ES, but the properties and substrate specificity of this protease have yet to be defined. Proteases may be involved in nutrition, but equally well may facilitate tissue disruption and penetration (Werb, Banda, McKerrow and Sandhouse, 1982; Dresden *et al.*, 1985). Enzymes may be necessary for parasite survival and are therefore potential targets for the immune response.

Enzymes of another class as yet unreported in ES are the glycosidases (Gentner and Castro, 1974). Glycosidases are present on the tegument and the caecal brush border of trematodes (Fujino, 1983). Glycosidase activity has been identified more recently in the lateral cords, (excretory apparatus) hypodermis and coelomycetes of five nematodes: *A. suum*, *T. canis*, *A. caninum*,

Dirofilaria immitis and *Trichuris vulpis* (Fujino and Ishii, 1986). Functionally, the glycosidases may be involved in digestion, alternatively they may be involved in degradation of glycoconjugates (Fujino and Ishii, 1986).

Allergens

Allergens are proteins or protein-bound substances which, by definition, bind reagins and promote reaginic antibody responses (Klein, 1982). Most environmental allergens are inhaled or ingested and are subsequently processed in the mucosal tissues close to the site of IgE synthesis (King, 1976). Purified allergens are commonly used for clinical diagnostic purposes in human medicine. Hypersensitivity to allergens can be detected by skin tests. Purified nematode allergens have been used in diagnosis of human Visceral Larva Migrans (Hogarth-Scott, 1967) and filariasis (Fujita and Tsukidate, 1980).

Extracts from plants or parasites are complex mixtures, and may contain multiple allergens. It is therefore essential that the latter should be purified for precise biochemical characterization. Further chemical and immunological characterization is conducted to permit differentiation between similar allergens from the same source. As yet, there are no distinctive differences between allergens and other antigens which fail to induce IgE antibodies, but there are a number of physicochemical properties common to many allergens. For example, allergens frequently have a molecular weight below 70,000 and most have a molecular weight between 15,000

and 40,000 (Klein, 1982). Furthermore, many allergens are polar compounds although there are exceptions to this generalization (Klein, 1982; Underdown and Goodfriend, 1969; Lind, 1985). The isoelectric points (pI) of most allergens fall within the rather narrow range of 3 to 6 (75% of known allergens) and 8 to 11 (25%) but only a very small number fall between these ranges (Lowenstein, Ipsen, Lind and Matthiesen, 1987). The most active allergens from ragweed, timothy grass, birch and alder tree pollens, together with the predominant cod fish, dust mite and animal dander allergens are acidic proteins with pI's between 4 and 6 (reviewed by King, 1976 and by Lowenstein, *et al.*, 1987; Hemmens, Baldo, Bass, Vak, Florraag and Elsayed, 1988; Haavik, Frostad, Paulsen and Wold, 1987). From the general physicochemical properties of allergens such as isoelectric point (pI), chromatographic mobilities and solubility in physiological buffers it has been suggested that most are probably globular proteins (Lowenstein *et al.*, 1987). Similar physicochemical observations have been made for parasite allergens (Hussain, Bradbury and Strejan, 1973; Jones, 1967; Wilson, 1967).

For example, a somatic allergen from *N. brasiliensis* was resistant to acid and alkali but not to heat or TCA treatments. Partial reductions in allergenicity were observed subsequent to periodate oxidation or proteolytic enzyme treatments (Wilson, 1967). Similar results were reported by Ambler and Orr (1972) when, after proteolysis of worm antigens with chymotrypsin or trypsin, where more than 50% of the protein present was digested,

allergenicity was only partially reduced. In view of these findings it was proposed that the allergenic epitope may comprise carbohydrate and/or carbohydrate-protein (Ambler and Orr, 1972; Wilson, 1967).

Although allergenicity has not been associated with any one physicochemical property, protein size and charge may influence the nature of the immune response produced (King, 1976). For example, the upper molecular weight limit for allergens is governed by the ability of soluble proteins to cross the mucosal barrier . The lower molecular weight limit for allergens and antigens alike is probably determined by the minimum structural complexity required to induce antibodies (Lowenstein *et al.*, 1987).

There are many reports that treatment with TCA significantly reduces the allergenicity of purified allergens (reviewed by Lowenstein et al., 1987). Therefore allergenicity is probably dependant upon protein conformation. Chemical treatments that affect protein stability and conformation may influence allergenicity (Lowenstein, 1987). Because of heterogeneity and lack of a general structure for allergens, it is not surprising that allergens express a variable stability to chemical, physical and enzymatic treatment (Lowenstein *et al.*, 1987). This heterogeneity is apparent when the stability of grass and tree pollens to physical or chemical treatments is examined. The main ragweed and timothy allergens were sensitive to low pH and denaturation (King, Nowman and Tao, 1974; Haavik *et al.*, 1987) but not to digestion with chymotrypsin or trypsin (King, Norman and

Lichtenstein, 1967). In contrast, the major cod allergen, Allergen M, was not affected by acid or heat denaturation but was sensitive to chymotryptic or tryptic digestion (Aas and Elsayed, 1969). Ryegrass and Birch tree pollen allergens were also sensitive to chymotryptic and tryptic digestion (Johnson and Marsh, 1966; Cesta and Ponterius, 1973).

There is considerable functional diversity among the allergens that have so far been identified. Some are enzymes like phospholipase A and hyaluronidase, others have reproductive functions, but, for the majority of allergens, their biological function remains undefined (Lowenstein, *et al.*, 1987). Allergens are frequently found among helminth somatic proteins and ES products.

Nematode allergens: Parasitic nematodes are complex organisms and it is therefore not surprising that one or more of their components stimulate IgE antibodies. Any one of a number of proteins expressing the correct physicochemical properties, and released at the appropriate site in the correct concentrations may promote IgE synthesis. Allergens have been identified in a large number of parasitic nematodes: *Acaris suum* (Hussain *et al.*, 1973), *A. lumbricoides* (Davidson, Baron and Walzer 1947) *Dirofilaria immitis* (Fujita, Ikeda and Tsukidate, 1979), *N. brasiliensis* (Jones and Ogilvie, 1967; Wilson, 1967; Petit, Pery, Luffau, 1980), *Trichostrongylus colubriformis* (Ford, 1971), *T. spiralis* and *T. canis* (Durham, Murrell and Lee, 1984; Santamarina, Leiro, Sanmartin, Urbeira, 1988).

Allergens from *A. suum* and *N. brasiliensis* have been studied most intensively and were demonstrable from all parasitic stages of both species (Bradbury, Percy and Strejan, 1974; Wedrychowicz, Bezubik and Krasuska, 1986). Such observations are not common; for example, only the adult stage of *D. immitis* contained demonstrable allergens (Ohara, Ikeda, Tani and Fujita, 1985). Three immunologically distinct allergens have been purified from adult *A. suum*, ASC-1 (Ambler, Croft, Doe, Gemmill, Miller and Orr, 1973), Allergen A (Hussain *et al.*, 1973) which is also referred to as ABA-1 (Kennedy, Tomlinson, Fraser and Christie, 1990), and one other less well defined allergen (Kuo and Yoo, 1977). ASC-1 is demonstrable in all stages of *A. suum* and is thought to play an important role in stimulating reagin production during the migratory stage of parasitic infection (Bradbury *et al.*, 1974). ASC-1, purified by a combination of gel-filtration and ion-exchange chromatography has a molecular weight of 17,000 to 19,000 by gel-filtration but is dissociated into two equal subunits of 8,200 MW when treated with SDS (Hussain *et al.*, 1973). Allergen A, which is present in both AWH and ES, is a glycoprotein (1.5% CHO) with a pI of 5.0-5.2. Allergen A and ABA-1 are physicochemically similar with the only real difference being that ASC-1 is a dimer.

Allergens from *N. brasiliensis* are less well defined, but three immunologically and physiologically distinct allergens were

detected in adult *N. brasiliensis* homogenate (Wedrychowicz *et al.*, 1986). There are many more uncharacterized high molecular weight allergens in *A. suum* perienteric fluid and *N. brasiliensis* homogenate (McWilliam, Stewart and Turner, 1987).

As already discussed, allergens are also found in ES. Yamada *et al.*, (1990) indicated that for a protein to be allergenic it should be secreted continuously. Wilson (1967) identified and partially characterized a 12,000 to 15,000 MW allergen from *N. brasiliensis* ES. This allergen had physicochemical properties similar to those already discussed for the *N. brasiliensis* AWH allergen (Ambler and Orr, 1976). In the latter study, the authors failed to determine the molecular weight of the allergen but it copurified with parasite haemoglobin and is, therefore, of relatively low molecular weight. It is likely that the ES and AWH allergens discussed here are one and the same. Minor differences in molecular weight may be explained by the distinct molecular processing mechanisms used by the parasite, for somatic and secreted proteins.

Recent studies with *Trichinella* species have identified immunological cross-reactivity between allergenic components of *T. spiralis*, *T. nelsoni* and *T. nativa* (Santamarina *et al.*, 1988). Further cross-reactions were observed between the allergens of *A. suum*, *T. canis* and *N. brasiliensis* (Mc William *et al.*, 1987). Antigenic similarity among diverse species may be explained by expression of common haptens. Phosphorylcholine is one such hapten which has been detected on somatic and released antigens of many

helminths (reviewed in Maizels and Selkirk, 1988; Uberia, Leiro, Sesanne and Reguairo, 1987; McWilliam, 1987). However, reaginic antibody for phosphorylcholine has not yet been detected *in vivo*. Similarities between allergens are not restricted to nematode allergens, but have also been observed amongst environmental allergens (Enberg, Leickly, McCullough, Bailey and Ownby, 1987; reviewed in Lowenstein *et al.*, 1987).

Intestinal Immunity to Nematodes

Intestinal helminthiasis is invariably associated with inflammatory changes in the gut (Cobden, Rothwell and Axon,1979). While tissue damaging activities or toxic products of the worms contribute to intestinal pathology, there is a considerable experimental evidence to suggest that inflammation is due to a specific immune response against the parasite (reviewed in Wakelin, 1978 and Miller, 1984). The inflammatory changes occurring in the intestine during helminth infection include: (i) inflammatory cell infiltration into the mucosa, predominantly with mast cells and eosinophils (ii) mucosal oedema, with leakage of plasma into the gut lumen; (iii) villous atrophy; (iv) goblet cell hyperplasia and mucous hyper-secretion; (v) disruption of epithelial integrity and (iv) increased peristalsis (reviewed in Miller, 1984). These immunopathological changes, while detrimental to normal intestinal function, may be beneficial in the long-term if they contribute to the expulsion of the parasite.

The immune reponse to nematodes has been reviewed and discussed at length elsewhere (Miller, 1984). For this reason, IgE and mast cells, which are the subject of this thesis will form the focus of this part of the introduction. However, our understanding of the roles of mast cells, IgE and the allergic response in immunity to nematodes is incomplete without first considering the immunoregulatory role of the T cell.

T cells and Parasite Immunity

T helper cells (T_H cells) are central to many immune responses, and play a pivotal role in inflammatory hemopoiesis and immunoregulation (reviewed in Miyajima, Miyatake, Schreurs, De Vries, Arai, Yokota and Arai, 1988). Their role in immunity to nematodes has been the subject of intense research. Most of these studies involve either immunocompromised or passively immunised laboratory animals. In neonatally thymectomized rats and mice, where infection with *N. brasiliensis* (Kelly, 1972; Mitchell, Hogarth-Scotti, Edwards and Moor, 1976; Jacobson and Reed, 1976) and *T. spiralis* (Wakelin, 1978) is prolonged, the absence of T cells is associated with a lack of mucosal immunity and the mucosal mast cell response is reduced or absent (Brown, Bruce, Manson-Smith and Parrott, 1981).

Vos, Ruitenberg, Van Baten, Elgersma and Kruizinga (1983) conducted a detailed study of *T. spiralis* infection in congenically athymic rats. Immunological staining failed to detect T cells in

the spleen, mesenteric lymph node (MLN) or Peyer's patch in these animals. Athymic rats retained their parasite burden for 87 days and mucosal mastocytosis was permanently delayed. In contrast, normal littermates expelled their parasite burdens by day 14, and responded with typical mast cell hyperplasia. Muscle digestion techniques revealed 33 times as many muscle larvae in the athymic animals as in the immunocompetent control mice (Vos *et al.*, 1983).

A similar study was performed a decade earlier with *N. brasiliensis* in congenically athymic nude mice (Jacobson and Reed, 1974). Athymic mice were more prone to chronic infection with *N. brasiliensis* and were unable to expel the parasite burden in their lifetime. In the same study, athymic mice were given thymus implants and mounted an effective immune response and expelled their parasite burden (Jacobson and Reed, 1974).

Katona, Urban and Finkelman (1988) investigated the roles of T_H cells in immunity to *N. brasiliensis* infection in mice. Immediately before infection, animals were given 0.5mg of anti-L3T4 monoclonal antibody intravenously. MMC proliferation and polyclonal IgE responses were almost totally ablated and parasite infection was greatly prolonged as a consequence.

Although the T cell plays a central role in the orchestration of the response, they are probably not the effector cells in their own right but act as regulatory cells. T_H cell function is mediated to a great extent by a large number of pleiotropic cytokines or lymphokines which are produced by sensitized T cells responding to

antigenic peptides in the context of Major Histocompatibility Complex (MHC) Class II antigens. Cytokines can influence virtually every aspect of leukocyte growth and differentiation. Their importance to the immune response is emphasised by the effect of cyclosporin A, an immunosuppressive drug which inhibits transcription of cytokine genes and which blocks immunological reactions, including rejection of transplants (reviewed in Miyajima *et al.*, 1988).

Murine T_H cells have been allocated to two distinct T cell subsets, T_{H1} and T_{H2}, according to function (Mossman, Cherwinski, Bond, Giedlan and Coffman, 1986). Both subsets secrete interleukin (IL) 3 and GM-CSF but each also expresses a unique profile of cytokines (reviewed in Miyajima *et al.*, 1988). T_{H1} cells secrete IL-2 and interferon gamma and are well suited to respond to intracellular infections with bacteria or protozoa. In contrast, T_{H2} cells produce IL-4, 5 and 6 cytokines that influence the allergic response (Mossman *et al.*, 1986; Miyajima *et al.*, 1988). Molecular cloning has permitted detailed study of the functions of cytokines and a considerable amount of information has now been accumulated regarding their biological role in health and disease.

IL-3 is a panhaemopoietic cytokine which also promotes mast cell growth and differentiation and the cloned molecule can support murine and rat, but not human, mast cell development from bone marrow stem cells *in vitro* (Valent, Besemer, Sillaber, Butterfield, Eher, Majdic, Kishi, Klepetko, Echersberger, Lechner and

Bettelheim, 1990). . Mesenteric lymph node (MLN) cells collected from parasitized rats, are a rich source of IL-3 (Haig, McMenamin, Redmond, Brown, Young, Cohen and Hapel, 1988) and conditioned media prepared from these cells are also capable of supporting mast cell growth (Haig, McKee and Jarrett, 1982).

Murine IL-4 augments the capacity of IL-3 to cause mast cell growth and differentiation, but is unable to maintain mast cell cultures on its own. The role of IL-4 in other species is less well defined (Miyajima *et al.*, 1988). In addition, IL-4 is involved in B cell activation and in isotype switching (Mossman *et al.*, 1986; Maggi, Franco, Del Prete, Tiri, Macchia, Parronchi, Ricci and Romagnani 1987). Lipopolysaccharide-activated murine B cells produce IgE and IgG_1 isotypes when stimulated with IL-4. The enhancing effects of IL-4 were negated by pre-treating cells with a specific anti-IL-4 monoclonal antibody (Mossman *et al.*, 1986; Snapper, Finkelman, and Paul, 1988) or by including interferon gamma in the cell culture (De France, Aubry, Rousset, Van Der Liet, Bonnefoy, Arai, Takebe, Yokota, Lee, Arai, De Vries and Banchereau, 1987; Hudak, Gollnick, Conrad and Kehry, 1987). IgE synthesis is enhanced when IL-5 is included in the system. However, on it's own IL-5 is unable to influence the isotype switch. IL-5 is also active in hematopoiesis, promoting eosinophil differentiation (Swain, Mackenzie, Dutton, Tonkonogy and English, 1988)

Based on the immunological responses observed after antigen challenge, it should be possible to predict which T cells were preferentially stimulated in the mouse (Swain *et al.*, 1988). The dramatic increases in concentration of serum IgE and numbers of intestinal mast cell and eosinophils observed would indicate that murine T_{H2} cells were active during intestinal nematodiasis. T_{H2} cells may, therefore, stimulate both mast cell and IgE responses. This is not unrealistic considering that the same T cell subset could, theoretically, control both responses. Whether similar divisions of T cell activity occur in other species is not yet known.

Mast Cells

Mast cells are found in the skin, upper and lower respiratory tract, gastrointestinal mucosa and in many connective tissues. They are usually in close proximity to peptidergic nerves (Stead, Tomioka, Quinonez, Felten and Bienenstock, 1987) or blood vessels. Histologically they possess a spherical nucleus and large numbers of secretory granules. The granules store vasoactive amines, proteoglycan and enzymes, some of which, when released during cell activation, promote local anaphylaxis. In addition to these preformed mediators, mast cells are also a source of newly generated membrane-derived mediators such as the leukotrienes (LT) prostaglandins, and platelet-activating factor (PAF) (Baird, Cuthbert and Pierce, 1985; Mencia-Huerta, Razin, Ringel, Corley, Hoover, Austen and Lewis, 1983; Moqbel, Wakelin, MacDonald, King,

Grencis and Kay, 1987). Lipid mediators such as PAF and LT can cause mucosal damage (Moqbel and Pritchard, 1990) and may act directly within the gut by inhibiting nematode motility (Douch, Harrison, Buchanan and Greer, 1983).

Mast cell populations have been subdivided into two distinct subsets on the basis of histochemical and physiological observations. This heterogeneity is most clearly defined in the rat. Connective tissue mast cells (CTMC) predominate in non-mucosal sites and atypical, or mucosal mast cells (MMC) are preponderant in mucosal tissues such as the intestinal lamina propria. Rat CTMC and MMC differ histochemically, biochemically and functionally (reviewed by Schick and Austen, 1987; Miller, King, Gibson, Huntley, Newlands and Woodbury, 1986).

Rat CTMC and MMC express variant granule proteases. Detection of distinct granule enzymes in CTMC and MMC has contributed to our understanding of mast cell heterogeneity and differentiation. These granules chymases have been purified (rat mast cell protease I (RMCP I) from CTMC, and rat mast cell protease II (RMCP II) from MMC) and their functional and biochemical properties characterized (reviewed by Woodbury, Le Trong, Cole, Neurath and Miller, 1989). The two granule chymases share amino acid sequence homology, substrate specificity, and three-dimensional configuration (based on amino acid homologies) (Woodbury, Everitt, Sanada, Katunuma, Lagunoff and Neurath, 1978; Woodbury and Neurath, 1981; Woodbury *et al.*, 1989). Despite a 75% overall sequence homology (Le Trong, Newlands, Miller, Charbonneau,

Neurath and Woodbury, 1988) RMCP I and II are distinguishable using immunological techniques (Woodbury, Gruzenski and Lagunoff, 1978; Gibson and Miller, 1986) and are functionally and biochemically distinct (Woodbury and Neurath, 1981).

The substrate specificity of RMCP I and II, although chymotrypsin-like, is more restricted because of a hydrophobic amino acid within the active site (Yoshida, Everitt, Woodbury, Neurath and Power, 1980). Both RMCPI and II are basic proteins, but this is especially so for RMCPI which binds more avidly to granule proteoglycan as a consequence. RMCPI can be detected still bound to proteoglycan after granule release (Schwartz, Reidel, Caulfield, Wasserman and Austen, 1981). This association may impose stoichiochemical restrictions on RMCPI (Le Trong *et al.*, 1987).

The *in vivo* functions of mast cell granule proteases remain undefined. Their presence within secretory granules suggests an extracellular role, possibly in the potentiation of mast cell-mediated anaphylaxis (Miller, Woodbury, Huntley and Newlands, 1983; Woodbury *et al.*, 1989). Initial studies suggest this may be the case since RMCP II, like RMCP I catabolizes type IV basement membrane collagen *in vitro* (Woodbury *et al.*, 1989). Experimentally induced anaphylaxis, during which there is significant sloughing of gut epithelium, is associated with massive

and instantaneous release of RMCPII into the intestinal lumen, and is followed by accumulation of RMCP II in peripheral blood and the accumulation of plasma proteins in intestinal perfusates (Miller *et al.*, 1986).

Mast cells can be activated via a number of pathways and activation via cell surface bound IgE has been intensively studied. Mast cells express high affinity IgE receptors (FcERI) on the cell surface (Sterk and Ishizaka, 1982; Lee, Sterk, Ishizaka, Bienenstock and Befus, 1985). The receptor is made up of three distinct peptides (Alcaraz, Kinet, Liu and Metzger, 1987) and binds the third constant Epsilon heavy chain domain (Burt, Hastings, Healy and Stanworth, 1987). When surface-bound IgE is cross-linked by allergen or anti-rat IgE, mast cells release their stores of biologically-active mediators (reviewed by Miller 1984 and Wasserman, 1983). Cross-linking of only a few IgE molecules on the cell surface is sufficient to cause cell activation and degranulation (Ishizaka, Jardieu, Akasaki and Iwata, 1987). Rat mast cells can also be activated via receptors for IgG_{2a} and by neuropeptides (Stead, Tomioka, Quinonez, Simon, Felten and Bienenstock, 1987).

IgE Antibody

By definition, allergenic proteins stimulate the production of IgE immunoglobulin and initiate allergic reactions. Rat IgE can be differentiated from the other rat immunoglobulin isotypes by its functional and molecular properties (reviewed in Bazin and Pauwels,

1982). IgE, as already indicated, is integral to mast cell activation and the allergic response (Reviewed by Miller, 1984). This will be discussed later under mast cells and nematode infection.

The molecular structure of IgE and the other immunoglobulin isotypes is similar (Bazin and Pauwels, 1982). IgE antibody comprises two heavy chains (E) and two light chains (K or L). Heavy chains of IgA, IgD and IgG are constructed from three constant and one variable domain. In contrast, Epsilon has an extra constant domain, CH4, as does the IgM heavy chain (μ). The extra domain may serve to restrict flexibility of the E chain in the hinge region. The E chain is heavily glycosylated and because of this and the extra domain, the MW of IgE is greater than those of IgG and IgA molecules. Bazin and Pauwels, 1982 estimated the MW of IgE from man to be 190,000. Bazin and Pauwels (1982) reported similar molecular weights for a number of rat myeloma IgE proteins. It is widely acknowledged that IgE is very susceptible to mild denaturing conditions (0.1M 2-mercaptoethanol or 56^{o}C for several hours) (Binaghi, Benacerraf, Bloch and Kourilsky, 1964). IgE treated in this manner undergoes irreversible changes in the CH_3 and CH_4 domains that are responsible for its reaginic activity (Tomioka and Ishizaka, 1971; Burt, Hastings, Healy and Stanworth, 1987). As a consequence, IgE loses many of its reaginic properties and physiological activities (Stanworth, Humphrey and Bennich and Johansson, 1968; Dorrington and Bennich, 1973).

The IgE isotype, like IgG_1 is susceptible to cleavage with papain, producing Fc (95,000 MW) and Fab fragments (52,000 MW). Two antigenic determinants D_E1 and D_E2 have been characterized on the Epsilon chain, both of which can be found on the Fc fragment (Johansson and Bennich, 1971).

Detection of IgE

There are three *in vivo* assays for the detection of IgE, the Prausnitz and Kutzner (PK), Passive Cutaneous Anaphylaxis (PCA), and Active Cutaneous Anaphylaxis tests (ACA). Prausnitz and Kutzner (1921) were first to observe that atopic human sera could sensitize the skin of normal individuals. Normal recipients, sensitized by intradermal injection with atopic serum respond with localised wheal and flare reactions after direct challenge of the sensitized site with allergen. In the PCA test which, in principle, is similar to the PK test, laboratory animal recipients are also sensitized intradermally but, after 24 to 48 hours, are challenged by intravenous injection of allergen and Evans blue dye. The extent of the dermal response is quantified by measuring the diameter of the lesion or by extracting the Evans blue which leaks from the circulation to be deposited in the tissue, and can be quantified spectrophotometrically (Nawa and Miller, 1979).

The ACA and PCA tests are similar except that for ACA, subjects are either sensitized by infection/exposure or by intradermal injection with lymphocytes harvested from atopic or sensitized compatible donors. Challenge is again intradermal for actively

infected or sensitized individuals or intravenously for laboratory animal recipients of intradermal lymph cells. In all three *in vivo* tests, the extent of the allergic response is proportional to the quantity of and titre of sensitizing serum or of locally produced IgE, and the quantity of challenge allergen.

Butchko, Aspinal and Smith, (1984) tested the specificity and sensitivity of the PCA and ACA tests. Prior intravenous injection of IgE myeloma protein blocked passive and active PCA sensitization to ovalbumin (Butchko *et al.*, 1984). The authors suggested that, with high concentrations of IgE, there is competition for $Fc_{\varepsilon}RI$ on mast cells, a theory originally proposed by Godfrey (1975). Furthermore, when sera with high titres of anti-ovalbumin IgE were mixed with sera from *N. brasiliensis* infected rats, significant reductions in anti-ovalbumin PCA titres were observed. It would appear that low titres of locally synthesized IgE are not detectable under such conditions (Butchko *et al.*, 1984; Bazaral, Orgel and Hamburger, 1973).

Hogarth-Scott (1973) examined PCA responses against AWH, using serum from rats immune to *N. brasiliensis*, before or after infection of recipient rats with the same parasite. Infection with *N. brasiliensis* reduced the PCA response significantly. It was suggested that circulating allergen, present during infection, may complex with mast cell-bound or circulating IgE, thus reducing titres of parasite-specific IgE (Hogarth-Scott, 1973). Furthermore, circulating allergen could trigger mast cell activity and explain why organs such as the intestine sometimes appear

refractile to homologous challenge (Befus, Johnstone, Berman and Bienenstock, 1982). Hogarth-Scott's (1973) results support such a theory and circulating IgE-allergen complexes have also been described clinically in food allergies (Reviewed by Carini, 1987).

In vitro tests for IgE, such as radioallergosorbent assay (RAST) and Radioimmunoassay (RIST) have virtually replaced the *in vivo* tests. RAST is a simple and reproducible technique for measuring specific IgE levels in which paper discs are coated with allergen. The discs are then incubated in test serum and, finally, with radiolabelled anti IgE. Unless individual allergens are purified first, the RAST technique will only measure IgE-specific for a variety of allergens in a complex mixture. The RIST test measures total IgE concentrations. Paper discs are coated with anti-IgE antibody and incubated first with test serum and then with radiolabelled anti-IgE. In both RAST and RIST test, the quantity of bound radiolabel is directly proportional to the specific IgE concentration and is usually expressed in units of IgE/ml by comparing with a reference IgE preparation of defined concentration.

Immunoregulation of IgE

Concentrations of IgE are normally low in serum but are raised in animals with helminth infections or allergy (Jarrett, Haig and Bazin, 1976). The capacity to develop and maintain persistently high IgE titres is genetically controlled (Reviewed by Katz, 1980).

IgE is of major significance in allergic reactions and Katz (1980) proposed that the pathological consequences of IgE were so severe that a strict immunoregulatory mechanism was required to protect the host. The latter point was emphasized with SJL mice that responded to conventional antigens with IgG antibodies but could not respond with IgE antibody to the same antigens (Levine, 1971). Supporting this hypothesis of strict immunoregulation (Katz, 1980), the IgE responses of high and low responder phenotype mice were enhanced after low doses of X-irradiation (Tung, Chiorazzi and Katz 1978; Okumura and Tada, 1971; Chiorazzi, Fox and Katz, 1976) or treatment with immunosupressive drugs such as cyclophosphamide (Mitchell, 1976). T-suppressor cells are more susceptible to the effects of cyclophosphamide and X-irradiation than their T-helper counterparts (Akasaki, 1987) and a reduction in suppressor cell activity could explain why the IgE antibody responses are enhanced after X-irradiation or cyclophosphamide treatment (Tung *et al.*, 1978; Katz, 1980). IgE reponses like those of other immunoglobulin isotypes may be linked to the MHC but subsequent immunoregulatory processes dictate the magnitude of those responses (Katz, 1980). The role of the MHC will be discussed more fully in the next section.

Immunoregulation of IgE by cytokines

The role of IL-4 in the induction of IgE responses has already been discussed but synthesis of IgE is also self-regulatory. For example, rat splenic or mesenteric lymph node (MLN) lymphocytes, treated with IgE *in vitro*, produce IgE-binding factors (Uede,

Sandberg, Bloom and Ishizaka, 1983) which, by binding to IgE, potentiate or suppress IgE synthesis (Hirashimi, Yodoi and Ishizaka, 1980). Rat IgE potentiating factor is produced by $W3/25^+$, T cells expressing Fc receptors for IgE. Their numbers increase during helminth infection (White, Macon, Williams, Galfre and Milstein, 1978). IgE-bearing B cells and plasma cells are the targets for these factors (Katona, Urban, Scher, Kanellopoulos-Langevin and Finkelman, 1983). IgE suppressive factor inhibits IgE synthesis by committed plasma cells and reduces the total proportions of IgE-bearing lymphocytes in splenic lymphocyte cultures (Uede, Huff and Ishizaka, 1984). IgE potentiating factor appears to function by enhancing the differentiation of IgE^+ B-cells (Ishizaka *et al*, 1985). IgE synthesis is enhanced when IgE potentiating factor is selectively formed (Ishizaka, *et al.*, 1987).

IgE binding factors have been purified from rat serum using IgE-sepharose. They are detected *in vitro* by their capacity to inhibit rosette formation of $FcER^+$ lymphocytes with IgE-coated erythrocytes (Yodoi, Hirashima and Ishizaka, 1980). The potentiating factor, has a molecular weight of 13,000 to 15,000 and binds to Concanavalin A and Lentil lectin but not to Peanut Agglutinin. Suppressive factor also has a molecular weight of 13,000 to 15,000 but does not bind to lectins (Yodoi *et al.*, 1980). Both factors share a common structural gene and therefore a common polypeptide chain (Ishizaka, *et al.*, 1987). They lack antigen specificity (Suemara, Kishimoto, Hirai and Yamamura, 1977). Biological activity is determined by post-translational

glycosylation and the only apparent difference between these antagonistic factors is the composition of their carbohydrate, demonstrable by their disparate lectin-binding properties. After neuraminidase treatment, potentiating factor assumes suppressive properties (Yodoi *et al.*, 1980).

IgE antibody synthesis can be influenced by special immunization regimes and adjuvants. Alum (aluminium hydroxide) and *Bordetella pertussis* adjuvants both enhance IgE antibody synthesis (Petillo and Smith, 1973; Smith, Petillo and Hwang, 1972; Jarrett and Stewart, 1972) and have been used to raise IgE antibody to antigens such as egg albumin, Keyhole Limpet haemocyanin (Jarrett and Smith, 1972) and the hapten dinitrophenol (DNP) (Kojima and Ovary, 1975). Even using IgE-potentiating adjuvants, IgE responses can be weak, short-lived and non-anamnestic (Petillo and Smith 1973). Complete Freund's adjuvant (CFA) suppresses IgE and enhances IgG antibody synthesis by promoting IgE suppressive factor production (Tung *et al.*, 1978; Hirashima, Yodoi, Huff and Ishizaka, 1981). In both cases, adjuvant induces macrophages and monocytes to release soluble factors that promote synthesis of IgE-binding factor (Yodoi *et al.*, 1980; Hirashimi, *et al.*, 1981).

Jarrett and Stewart, (1974) demonstrated that the quantity of sensitizing antigen can influence the nature of the humoral response. Low rather than high concentrations of sensitizing antigen were required to stimulate an anamnestic reaginic antibody response. Similar findings were documented by Strejan, Rabbera, White and Surlan (1977) who immunized rats with *Ascaris* allergen

conjugated with high or low numbers of DNP (Dinitrophenol) haptenic molecules. After immunisation with heavily substituted allergen, rats produced a short-lived anti-DNP IgE antibody response, and failed to produce anti-carrier IgE antibodies. In animals immunised with a lightly substituted allergen conjugate, strong IgE responses were observed to both hapten and carrier (Strejan *et al.*, 1977).

Antibody Repertoire and the MHC

It is widely accepted that genetics play a substantial role in the predisposition to allergy (Marsh, Hsu, Roebber, Erhlich, Kautzby, Friedhoff, Meyers, Pollard and Biras, 1982). Breeding experiments have established that the Class II Major Histocompatibility Complex (MHC) plays an integral role in immunoregulation, influencing the immune repertoire and the magnitude of the response (Katz, 1980). Existing information suggests that the MHC exerts a strong influence on the protective response to experimental parasitic infection in mice (Wassom, Brooks, Cypess and David, 1983; Wassom, Bradford, Brooks, Cypess and David, 1983).

Preliminary studies of *Ascaris* in man indicates great variability between individuals in the IgG and IgE responses (Kennedy *et al.*, 1990). The MHC has been implicated as an important factor in heterogeneity of the human response.

Furthermore, MHC-imposed genetic restrictions influence the IgE response to parasitic allergens (Tomlinson, Christie, Fraser, McLaughlin, McIntosh and Kennedy 1989; Christie, Dunbar, Davidson and Kennedy, 1990).

The effects of the MHC on the immune antibody repertoire were addressed more extensively by studying *Ascaris* infection in mice with defined MHC haplotypes. Animals expressing the same MHC haplotype recognized the same antigens and the same allergens, despite their disparate genetic backgrounds (Tomlinson *et al.*, 1989). Using H-2 congenic mice, the region within the MHC controlling responsiveness was defined as the I-A locus (Kennedy, Fraser and Christie in press). It is, therefore, apparent, that the MHC defines which parasite epitopes are immunogenic.

However, *Ascaris* infection in mice can be argued as inappropriate for studies of IgE for two reasons, first because the mouse is a novel host and second because parasite development is arrested and the life cycle is not completed (Kennedy *et al.*, 1990). To clarify the role of the MHC and antigen recognition Kennedy, McIntosh, Blair and McLaughlin (1990b) studied *N. brasiliensis* infection in rats, a natural host-parasite system. The *Nippostrongylus*-specific antibody (IgG) responses of 10 rat strains were investigated by immunoprecipitation and SDS-PAGE. MHC-restriction of the rat antibody repertoire was evident, to the extent of limiting recognition of a 98,000 MW protein. It was

concluded that MHC restriction of the antibody repertoire is probably commonplace during parasitic infections. Furthermore it explains the heterogeneity of the human antibody response after parasite infection (Kennedy *et al.*, 1990b).

Kinetics of IgE production during nematode Infection

To help monitor nematode-induced potentiation of the IgE response, animals were first immunized with novel antigens in *B. pertussis* and alum adjuvants (Smith, Petillo and Hwang, 1972; Petillo and Smith, 1973; Jarrett *et al.*, 1976). Serum IgE concentrations directed against the immunizing novel antigens rise dramatically during nematode infections (Orr and Blair 1969; Ogilvie and Jones, 1973; Jarrett *et al.*, 1976) and remain high for long periods after parasite expulsion (Reviewed by Kojima, Yokogawa and Tada, 1972; Jarrett and Ferguson, 1974).

In normal Wistar rats, serum contains 1.28μg IgE/ml, and after infection with *N. brasiliensis*, concentrations of IgE rise quickly, with the most rapid rate of increase observed between 8 and 10 days. Concentrations of IgE in serum are maximal (247 μgs/ml) at 12 to 14 days p.i. and subsequently decline, at first rapidly and then more slowly, with concentrations remaining at 25 μgs/ml on day 80 (Jarrett and Stewart, 1973). All parasitic stages of *N. brasiliensis* can potentiate IgE synthesis against an immunizing antigen (Jarrett and Stewart, 1973).

Helminth infection initially provides a polyclonal stimulus to IgE-B cells where *de novo* IgE synthesis is not observed (Jarrett and Ferguson, 1974; Turner, Fisher and Holt, 1987). This phenomenon appears to be a T cell controlled non-specific boosting of ongoing IgE responses by B cells already committed to IgE synthesis (Turner *et al.*, 1987). T cells isolated from infected animals selectively enhanced differentiation of IgE-bearing cells from normal donors (Suemara and Ishizaka, 1979).

Attempts to demonstrate parasite-specific IgE in serum by PCA, prior to the expulsion of *N. brasiliensis* have failed (Jarrett, *et al.*, 1976; Allan and Mayrhofer, 1981; Befus *et al.*, 1982). However, after secondary infection, total and parasite-specific IgE followed similar kinetics, both reaching a peak 6 days after reinfection; parasite-specific IgE titres constituting a greater proportion of the total (376 μgs IgE/ml) (Jarrett and Stewart, 1973).

The Mast Cell Response to Nematode Infection

Histological studies have demonstrated a dramatic intestinal mastocytosis during nematode infection (Miller and Jarrett, 1971; Askenase, 1977). Mast cell numbers are maximal 10 to 14 days after infection with *N. brasiliensis* (Miller and Jarrett, 1971) and parasite expulsion is observed around days 12 to 15 (Jarrett, Jarrett and Urquhart, 1968). At the same time mast cell activity is demonstrable histologically (Miller, 1971) and through the

release (into blood) of RMCP II which is an excellent functional marker of mast cell activity (Miller *et al.*, 1983; Woodbury, Miller, Huntley, Newlands, Palliser and Wakelin, 1984). Because of this temporal relationship between mastocytosis and parasite immunity the significance of mast cells during nematode infection has been studied intensively. However, there is considerable variation between the onset of mastocytosis and worm expulsion (Askenase, 1980; Miller, 1984) and this is still a controversial issue. Nevertheless, primary worm expulsion in both rats and mice is associated with the presence of mucosal mast cell protease in blood (Woodbury *et al.*, 1984; Huntley, Gooden, Newlands, MacKellar, Lammas, Wakelin, Woodbury and Miller, 1989).

Rats immunized by infection with *N. brasiliensis*, when challenged intravenously with parasite homogenate (King and Miller, 1984), undergo systemic anaphylactic shock. The shock organ is the gut, and anaphylaxis is associated with massive epithelial shedding and leakage of plasma proteins into the intestinal lumen. At the same time RMCP II is detectable both in the intestinal lumen and in blood (King and Miller, 1984; King, Miller, Newlands and Woodbury, 1985).

A similar but less pronounced intestinal anaphylaxis is observed when parasites enter the immune gut (Miller *et al.*, 1983; Moqbel *et al.*, 1986) and is accompanied by the almost immediate expulsion of the challenge infection, a process which is frequently referred to as rapid expulsion (RE). In immune rodents, RE occurs within 15-30 minutes of direct infusion of parasites into the gut, or

between 4 and 48 hours after entering the stomach in immune sheep (Russell and Castro, 1979; Miller, Huntley and Wallace, 1981; Huntley, Gibson, Brown, Smith, Jackson and Miller, 1987). Because RE in the rat involves a concomitant release of RMCP II, both mast cells and IgE are thought to be involved (Miller, Woodbury, Huntley and Newlands, 1983). Rapid expulsion of *N. brasiliensis* and *T. spiralis* in rats and *Haemonchus contortus* in sheep is associated with the release, into blood, of RMCP II and sheep mast cell protease respectively (Miller *et al.*, 1983; Moqbel *et al.*, 1986; Huntley *et al.*, 1987).

The involvement of mast cells in rapid expulsion was endorsed in studies with corticosteroids and inhibitors of vasoactive amines. Expulsion of *N. brasiliensis* was delayed when rats were treated with inhibitors of histamine and 5,hydroxytryptamine (Giertz, Glanzmann and Keller, 1970; Murray, Smith, Waddell and Jarrett, 1971). Furthermore, short-term corticosteroid treatment suppresses RE in immune animals (reviewed by Miller, 1984). This is associated with a selective depletion of mast cells (and globule leukocytes) (Jarrett, Jarrett, Miller and Uquhart, 1967; Miller, Jackson, Newlands and Huntley, 1985) and of RMCPII from the mucosa and with total suppression of intestinal anaphylaxis (King *et al.*, 1985). Similar results were reported in sheep immunized by infection with *H. contortus* and treated with corticosteroids (Miller *et al.*, 1985). It was also apparent that mucus-trapping was significantly reduced after corticosteroid treatment (Miller and Huntley, 1983) although mucus-trapping is not believed essential for RE (Miller *et al.*, 1981).

Scope of the Thesis

The aims of the experiments described in this thesis are to define, in more detail, the specificity of IgE responses against *N. brasiliensis* and to examine other parameters of IgE biology during intestinal nematodiasis. Also of interest is the relationship between mast cell granule proteases and IgE because of the reported internalization of IgE by intestinal and pulmonary mucosal mast cells (Mayrhofer, Bazin and Gowans, 1976). Finally, the distribution of IgE-bearing cells is analyzed during the course of infection.

CHAPTER 2

MATERIALS AND METHODS

PARASITOLOGICAL TECHNIQUES

The Parasite

A strain of *N. brasiliensis* obtained from the University of Glasgow was maintained at the Moredun Research Institute (MRI) and passaged in random-bred Wistar rats. Adult worms (L5) were harvested on day 7 of infection for the preparation of adult worm homogenate (AWH). Several different strains of 10 to 15 week old male or female rats, bred at the MRI, were used for all experiments (unless stated otherwise) and were given water and a standard pelleted diet *ad libitum*

Faecal Egg Cultures

Six days after infection with 3000 *N. brasiliensis* larvae, rats were moved into a wire-bottomed metabolic cage for 24 hours. Faeces were collected onto wet paper towels and mixed with an equal volume of washed, gas adsorption grade charcoal (BDH, Poole, U.K.). Faecal cultures were as described in "Handbook of *Nippostrongylus brasiliensis*" (Kassai, 1982) but without using sponge discs. Briefly, the faecal mixture was smeared onto wet fiter paper discs (Whatman No. 1; 5.5cm diameter), placed in petri-dishes and stored in a sealed humid chamber at 26^{o}C.

Harvesting larvae

After a minimum of 7 days, third-stage larvae (L_3) were harvested by adding warm (37°C) tap water to the petri-dish and were incubated at 37°C for 5 minutes before decanting into a filter funnel lined with porous paper (K-DEX, Kleenaroll Ltd, London). Viable larvae were collected at the bottom of the funnel after one hour and were washed several times in PBS containing 100 units/ml each of penicillin and streptomycin and counted under a dissecting microscope. Rats were inoculated under anaesthesia by subcutaneous injection in the flank with *N. brasiliensis* larvae. Primary infection was usually with 3000 L_3 and if required, secondary and tertiary infections were also given.

Faecal Egg Counts

Faecal egg counts were performed by Dr. F. Jackson of the MRI (Jackson, 1974). Briefly, the faecal sample was emulsified in 10 ml water/gram of faeces and passed through a mechanical sieve with 1 mm^2 mesh. The filtrate was centrifuged at 1000 rpm for 1 minute in cellulose acetate tubes. The pellet was resuspended in saturated NaCl (10 ml) and centrifuged as before. The supernatant was collected and restored to 10 ml with saturated NaCl and the number of eggs were counted, multiplied by ten and expressed per gram of faeces.

Worm Burdens

The small intestine was opened longitudinally, placed in surgical gauze and suspended in PBS for 2 hours at 37°C. Worms migrated through the gauze and were collected and counted under a dissection microscope. Although 90% of the adult worms moved through the gauze, a small proportion remained associated with the gauze bag or the intestine and their numbers were defined by examination under a dissection microscope. Adult worms were harvested for homogenization using the same procedure.

COLLECTION OF TISSUES

Preparation of Peripheral Blood Leukocytes

Blood was collected from the carotid artery into heparinized (25 units) universal containers by exsanguination under deep ether anaesthesia. The tubes were centrifuged at 300 g for 6 minutes and the buffy coat was removed and treated with red blood cell lysis buffer (see below).

Lysis of Red Blood Cells with Tris-Buffered Ammonium Chloride

Tris-buffered ammonium chloride was prepared from stock solutions A and B.

A) 0.16 M NH_4Cl (8.3 g/litre)

B) 0.17 M Tris, pH 7.65 (20.6 g Tris base adjusted to pH 7.65 with HCl and made up to 1 litre).

90 ml of 0.16 M NH_4 Cl and 10 ml of 0.17 M Tris pH 7.65 were adjusted to pH 7.2 with HCl. 40 mls of Tris buffered ammonium chloride was added to 10 ml heparinised blood and allowed to stand for 3 minutes. Cells pelleted by centrifugation at 300 g for 5 minutes, were resuspended in 10 ml PBS and heparin (10 units/ml) and the procedure was repeated. Peripheral blood leukocytes (PBL) were resuspended and washed twice in RPMI with 10% heat-activated fetal calf serum (FCS).

Peritoneal Lavage

Rats were killed by exsanguination under ether anaesthesia, from the carotid artery before intra-peritoneal injection with 20 mls of Earle's basic salt solution containing 0.5% bovine serum albumin (BSA, Sigma, Poole, U.K.). Earle's solution was made up as follows:-

A) $CaCl_2$ (BDH), (2.68 g in 200 mls of distilled water)
B) $MgSO_4$ (BDH), (2 g in 200 mls distilled water)
C) NaCl (BDH), (468 g in 400 mls distilled water)
KCl (BDH), (4 g in 100 mls distilled water)
NaH_2 PO_4 (BDH) (1.45 g in 100 mls distilled water)

Solutions A, B and C were mixed and heparin was added to produce a final concentration of 10 units/ml.

After intra-peritoneal injection of the above solution, the peritoneal cavity was massaged gently for 2 minutes before the solution was recovered through an incision in the abdominal wall.

Harvesting of Bone Marrow Cells

The femur was stripped of soft tissue and the head of the femur was removed using bone forceps and the bone shaft was perfused with 5 ml RPMI containing 20% FCS. The perfusate was aspirated gently several times through a 19 gauge needle to create a single cell suspension.

Viability Counts

The proportion of bone marrow, peritoneal lavage and peripheral blood cells still viable after harvesting was determined by dye exclusion. Cells were suspended at 1 x 10^5 cells/ml in 0.1% nigrosine (BDH) in PBS and then counted using an improved Neubauer haemocytometer. The proportion of viable cells was expressed as a percentage of the total cells counted and the viability of cell preparations used in all experiments was always greater than 95%.

Cytocentrifuge Preparations

Cytospin chambers (Shandon, Southern Instruments Ltd., Runcorn, U.K.) were loaded with 100 μl of cell suspension containing 1 x 10^4 cells/ml in RPMI 10% FCS, and centrifuged for 5 minutes at 600 g. Slides were air-dried and fixed with 4% paraformaldehyde in PBS at 45^{o}C for 60 minutes. Slides fixed in this way were washed four times in PBS before immunocytochemical staining and mounting in citifluor for analysis by UV microscopy. Alternatively, slides were fixed and stained with Leishman's stain (BDH, Poole, U.K.) and then analysed by light microscropy.

Leishman's Stain

Cytospin preparations were fixed and placed for 2 minutes in undiluted Leishman's stain (BDH). Slides were rinsed with cold tap water and then left for a further 10 minutes in diluted Leishman's stain (50% water: 50% Leishman's stain). Slides were rinsed well in water, air-dried, and finally mounted with 'Coverbond' (BDH, Poole, U.K.)

ANTIGEN PREPARATION

Adult worms were washed in six changes of PBS containing penicillin and streptomycin (100 units/ml each) and total worm numbers calculated by the method of Ogilvie, (1967). Briefly, the packed volume was measured after centrifugation at 200 g for

10 minutes (1ml packed volume contains approximately 4000 worms). Worms were then homogenized by mechanical disruption using a Potter homogenizer (F. T. Scientific, Tewkesbury, U.K.). For PCA analysis and for electrophoresis and Western blotting, adult worm homogenate (AWH) preparations were centrifuged at 6,500 g for 1 and 4 minutes respectively in an Eppendorf 5414 centrifuge. All steps were conducted at 4^{o}C or on ice.

PROTEIN ESTIMATION

Protein concentrations were measured by a colourimetric reaction using the bicine choninic acid (BCA)-Protein assay (Pierce Chemical Company, Rockford, Illinois, USA.) read at 562nm in a PYE-Unicam spectrophotometer (Cambridge, UK.) with a one-centimetre path length. Bovine serum albumin with an extinction coefficient of 0.6 was used to plot a standard curve for each assay and protein concentrations were estimated from this curve.

Immunoglobulin concentrations were determined by measuring absorbance at 280nm in quartz cuvettes. A proportional relationship exists between absorbance and protein concentrations, an immunoglobulin solution at 1 mg/ml having an absorbance coefficient of 1.39.

SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

A discontinuous mini-gel system (Mini-protean II, Bio-Rad Hemel Hempstead, U.K.) with the buffer system of Laemmli (1970), was used for analysis of protein samples. Gels were cast and run following the method recommended by Bio-Rad. Briefly, 0.75 mm thick slab gels, composed of a homogeneous 12% or 15% resolving gel and 4% stacking gel were used. A 4% acrylamide stacking gel solution containing 0.1% N,N, methylenebisacrylamide, 0.25% SDS in 0.125M Tris-HCL buffer, pH 6.8, and a 12% or 15% acrylamide resolving gel containing 0.33% N,N,-methylenebisacrylamide and 0.5% SDS in 0.125 M Tris-HCl buffer pH 8.8 were polymerized by the addition of 200μl of a 10% ammonium persulphate solution and 20ul of N,N,N N'-Tetramethylethylene diamine (Temed), (BDH., Poole, U.K.).

Gels were cast using 10- or 15-lane combs for loading and analysis of multiple protein samples, or, alternatively, by using a preparative gel comb for analysis of a single protein sample by Western blotting.

Samples for electrophoresis were diluted 1:1 in SDS-PAGE sample buffer: 10% glycerol, 2% SDS, 0.1% w/v Bromophenol blue in 63 mM Tris-HCl pH 6.8. 2-mercaptoethanol was added to the otherwise complete sample buffer, to a concentration of 10% V/V immediately before use. Samples were heated in a boiling water bath for at least 2 minutes before immediate loading onto a gel.

When non-reducing conditions were employed samples were diluted in sample buffer as above, but with the omission of 2-mercaptoethanol. Non-reduced samples were heated for 30 seconds in a 60°C water bath.

Gels were loaded and run for approximately 55 minutes at room temperature at a constant 200 volts. Power was supplied by a Pharmacia Power Supply EPS 500/400 power pack. Running buffer, 0.2 M glycine, 0.25 M Tris and 0.1% SDS, was freshly prepared before each run. Molecular weight markers were included in each gel analysis.

The molecular weight markers used were the following:- 21,500 MW (trypsin inhibitor), 30,000 MW (bovine carbonic anhydrase), 45,000 MW (ovalbumin), 66,200 MW (bovine serum albumin) and 97,400 (phosphorylan B) (Bio-Rad, Hemel Hempstead U.K.) or 12,300 MW (equine cytochrome c) 17,200 MW (myoglobin) 30,000 MW (carbonic anhydrase), 42,700 (hen egg ovalbumin), 66,200 MW (bovine serum albumin) and 76-78,000 MW (hen egg ovotransferrin) (B.D.H. Poole, Dorset, U.K.).

Fixing and Staining of Gels

Gels were fixed overnight with 30% methanol and 10% trichloroacetic acid and stained for 30 minutes with 0.1% W/V Coomassie blue in 50% methanol and 20% acetic acid. Gels were destained in successive changes of 45% methanol and 20% trichloroacetic acid.

WESTERN BLOTTING

The procedure, originally described by Khyse-Andersen (1984), was a semi-dry system and the apparatus constructed at the MRI is based upon the design of Kyhse-Andersen (1984). The technique involves the preparation of a filter paper - nitrocellulose membrane - polyacrylamide gel - filter paper sandwich to which a current is applied by means of two solid graphite electrodes.

Briefly, five sheets of Whatman chromatography paper number 3 (Whatman, Maidstone, U.K.) and a sheet of nitrocellulose with 0.2 μm pore size, (Schleicher and Schull, Dassel, West Germany) were trimmed to the appropriate size. Two sheets of chromatography paper were soaked in anode buffer 1 (0.3M Tris solution in 20% methanol, pH 10.4) and placed on the anode. One sheet of paper, soaked in anode buffer 2 (25 mM Tris solution in 20% methanol, pH 10.4) was placed on top of the other two wet sheets. The nitrocellulose membrane was soaked in water and then placed on top of the 3 layers of paper. The gel was placed carefully on the nitrocellulose membrane and the sandwich was completed by the addition of two further sheets of paper soaked in cathode buffer (25 mM Tris, 40 mM 6 amino-n-hexanoic acid solution, 20% methanol pH 9.4).

The transfer cell was completed by placing the cathode plate on top and a current of 0.8 mA/cm^2 was applied from a Bio-Rad power pack (model 160/1.6) for 90 minutes.

Remaining protein-binding sites on the nitrocellulose membrane were blocked by incubation in PBS blocking buffer containing 1.0M NaCl, 1mM EDTA and 1%V/V Tween 20 and 10% egg albumin (Sigma Chem. Co. Poole, U.K.) at room temperature, with agitation, for four hours. Blots were subsequently probed with immunological reagents also diluted in this blocking buffer. Between each step, blots were washed six times for five minutes each, in blot wash buffer followed by one 20-minute wash.

ELECTROELUTION OF PROTEINS FROM SDS-PAGE

Proteins separated by SDS-PAGE for electroelution were identified by staining adjacent strips of a preparative gel with Coomassie blue. Proteins were electroeluted using the Bio-Rad electroelution apparatus and following the method recommended by the manufacturer. Briefly, the excised band was chopped into $2mm^3$ pieces and loaded, in electrophoresis buffer, into the electroelution tube. The tank was filled with the same buffer and the apparatus assembled. A current of 10mA per tube was applied for 3 hours. After elution, the apparatus was dismantled and the eluted proteins collected and stored at $-20^{o}C$.

DOUBLE DIFFUSION AND IMMUNOELECTROPHORESIS

Double diffusion plates were prepared by dissolving 1% Agarose (BDH, Poole, U.K.) in PBS, pH 7.4, containing 0.01% sodium azide in a boiling water-bath. Fourteen ml of this solution were poured onto a clean glass plate 9 cm by 8.5 cm and six wells were cut around a central well using a punch and template (LKB, Broma, Sweden). Samples (10 μl each) were applied to the surrounding wells whilst an antibody sample was loaded in the central well. The plates were then incubated in a humid chamber for 18 to 48 hours.

Gels transferred to 'Gel-bond' (FMC Corporation, Bio-products, Rockland, U.S.A.) were pressed dry under absorbent paper, washed in PBS pH 7.0, for two hours and again pressed dry. This process was repeated four times. Finally, the gel was air-dried under a fan and stained with Coomassie blue stain.

Immunoelectrophoresis (IEP) plates were prepared in a similar manner to those for double diffusion, but with the exception that the agarose was dissolved in 0.1M tris barbiturate buffer pH 8.8. Six wells were excised 1cm apart, 2.5cm from the cathode end of the gel and were loaded with samples of 10ul. Gels were then placed in an LKB Multiphor II Electrophoresis unit. The same 0.1M tris-barbiturate buffer was used for both anode and cathode reservoirs. Bromophenol blue (1 μl) was

applied adjacent to and parallel with the wells to act as a marker. A current of 100 V was supplied by an LKB 2197 power pack, until the marker dye had migrated to within 1cm of the anode terminal. Troughs were excised between the wells and loaded with 100ul each of antisera. The gels were then incubated, washed and stained as described for double diffusion.

SEPHAROSE 4B IMMUNOSORBENTS AND COLUMN CHROMATOGRAPHY

Preparation of affinity columns

Coupling of protein to sepharose 4B beads was performed following the procedure recommended by the manufacturer (Pharmacia Ltd., Milton Keynes, U.K.). Briefly, proteins to be coupled were dialyzed against two changes of 0.1M $NaHCO_3$, pH8.3, containing 0.5M NaCl, for 16 to 18 hours. One gram of dry CNBr-activated sepharose 4B was used for every 5mg of protein. Sepharose was swollen in, and washed with 1mM HCl, pH3, and then with activation buffer 0.1M $NaHCO_3$, pH8.3, 0.5M NaCl. The activated gel and the protein to be coupled were mixed gently for two hours at room temperature. Coupling efficiency was calculated by measuring the optical density of the supernatant and was deemed successful if greater than 80% of the protein was bound to sepharose. The gel was then washed with 40ml of activation buffer per ml of swollen gel and then with 200ml 0.1 M Tris-HCl pH8, overnight. The mildly alkaline conditions in the Tris-HCl buffer are optimal for the hydration

of any remaining active sites. Successive high ("0.1M Tris-HCL buffer pH8 + 0.5M NaCl") and low (0.1M Sodium acetate pH4 + 0.5M NaCl) pH washes were subsequently performed to remove unbound materials from the gel.

Column Chromatography

Affinity chromatography was performed either at room temperature using an FPLC system (Pharmacia Ltd. Milton Keynes, England) or at 4°C using a peristaltic pump (Watson and Marlow), an Isco UA5 chart recorder with a multiplexer-expander and an MSE model 328 fraction collector (MSE). In both systems, absorbance readings were made at 280nm in continous flow cells. All column chromatography was carried out using Amicon, Wright, or Pharmacia columns.

Loading and Elution of Affinity Columns.

All columns were equilibrated in PBS, with 0.5M NaCl, 0.01% Na N_3, pH 7.5 at a linear flow rate of 0.1ml/min, and were loaded at the same flow rate. Bound material was eluted with 0.2M Glycine, pH 2.5 with 6M Urea at 0.25ml/min, and neutralized immediately with 1.0M Tris-HCl buffer pH 8.5. Eluate was then dialysed at 4°C against three 1 litre changes of running buffer for 1 hour each and against one 5 litre change

overnight. Eluate samples were concentrated by negative-pressure thimble dialysis (Sartorius, Gottingen, Germany) and stored at -20°C until tested by double diffusion and Western blotting.

DETECTION OF IgE BY AUTORADIOGRAPHY

For the detection of rat IgE using radiolabelled murine monoclonal anti-rat IgE (MARE-1), Serotec, Bicester, U.K.), MARE-1 (25 μg) was mixed with 100 μCi ^{125}I and two Iodo-beads (Sigma, Poole, U.K.) for 10 minutes at 4°C. Saturated tyrosine (10 μl) was added to give a final solution of 10% by volume. After a further 10 minutes 20 μl saturated sucrose was added and the solution was fractionated on Sephadex G25 packed in a PD10 dissposable column (Pharmacia, Milton Keynes, U.K.). Labelled fractions were pooled and stored at 4°C until required. Western blots probed with radiolabelled antibody were dried at room temperature and mounted on blotting paper. Radiographs were exposed in a radiographic casette with intensifying screen using Kodak X-omat AR film (Kodak Ltd., Hemel Hemstead, U.K.). Film was developed after 2-4 weeks exposure at -60°C.

PREPARATION OF IMMUNOLOGICAL REAGENTS

Purification of IgG_{2a} from Normal Rat Serum

The method used was essentially that published by Bazin, Querinjean, Beckers, Heremans, and Dessy, (1974). Normal rat serum was diluted 1:1 with PBS and subsequently with an equal volume of ice cold, saturated ammonium sulphate solution, added dropwise, over a period of twenty minutes. Samples were stirred for 16 hours at 4°C to allow equilibration between soluble and insoluble material. Precipitated material was collected by centrifugation and washed twice with a 50% saturated ammonium sulphate solution by centrifugation at 200 g. for 10 minutes using a MSE Chilspin 100 centrifuge.

The precipitate was redissolved and dialysed against five 1 litre changes of 0.05M Tris-HCl buffer, pH8.0. One hundred milligram samples of protein were applied to a DEAE Sephadex A50 column at 4°C. Bound material was eluted using the same buffer, but with the inclusion of 0.05M NaCl. The ascending fraction of the first peak was collected and concentrated by negative-pressure thimble dialysis and tested by immunoelectrophoresis (IEP).

Purification of IgE

Anti-rat IgE raised in goats by immunization with rat IgE myeloma IR162, was kindly supplied by Dr. Elizabeth Hall of the Glasgow Veterinary School. The specificity of untreated goat anti-rat IgE was defined by immunodiffusion (Fig. 2.1). To remove anti-light chain antibody, goat anti-rat IgE (G-anti-IgE) antiserum was cross-adsorbed against rat IgG_{2a} bound to sepharose 4B and tested again by immunodiffusion (Fig. 2.2). Specific goat anti-rat IgE immunoglobulins were subsequently purified by immunoaffinity on an IgE myeloma (IR2)- sepharose column supplied by Dr. ELizabeth Hall. Specificity was confirmed by immunodiffusion (Fig. 2.3).

Two milligrams of cross-adsorbed and affinity-purified goat anti-rat IgE, were coupled to one gram dry weight of CNBr-activated sepharose. This immunoabsorbant was used to purify IgE from myeloma ascitic fluid and from hyperimmune serum (HIS). Affinity purifications were performed at room temperature using FPLC. In order to minimize non-specific binding, samples were applied to a mouse column containing Sepharose-4B only. The flow through was immediately applied to the anti-rat IgE affinity column and IgE was eluted with 0.2 M Glycine/6 M urea, pH 2.5. Alternative eluents such as; 0.2 M Glycine buffer, pH 2.5 and phosphate buffered saline with 6 M urea, pH 7.0, were also tested but without success. Eluates were neutralized and dialysed as already described, and stored at -20^{o}C. IgE purified from 5ml of Hooded Lister HIS was

Figure 2.1.

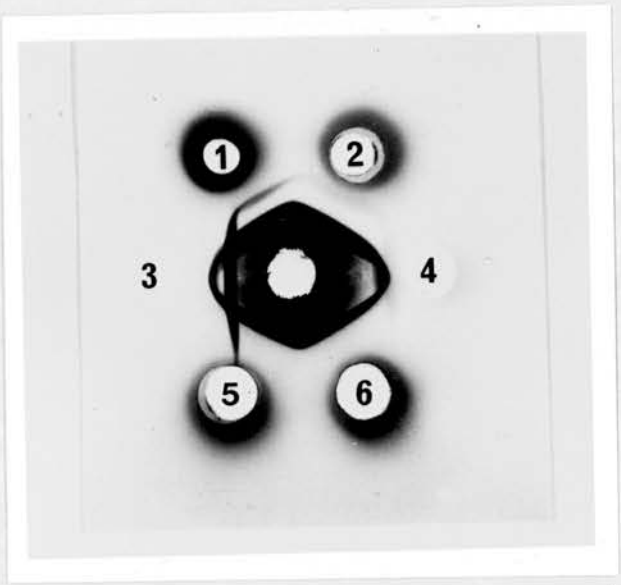
Specificity of untreated Goat anti-rat IgE: The central well contained 10 μls of G-anti-IgE and wells 1-6 were loaded as follows:- (1), hyperimmune serum; (2) LOU primary infection serum; (3), Myeloma rat IgE, IR2 ascitic fluid (1mg/ml); (4), Myeloma rat IgE, IR162 ascitic fluid (1mg/ml), (5), Normal LOU serum, and (6) normal F344 serum.

Figure 2.2.

Specificity of cross-adsorbed Goat anti-rat IgE (1 mg/ml - centre well). The loading of wells 1-6 was as described in Fig. 2.1.

Figure 2.3.

Specifity of cross-adsorbed, affinity purified Goat anti-rat IgE antbody (1 mg/ml - centre well). The other wells (1-6) were loaded as described in Fig. 2.1.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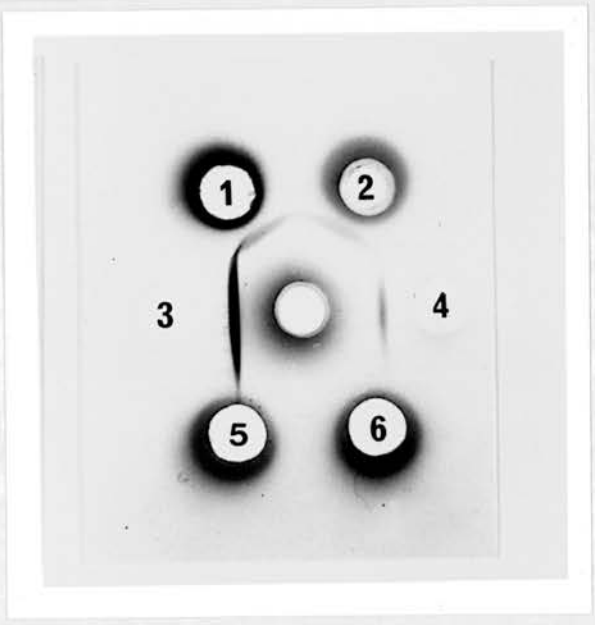
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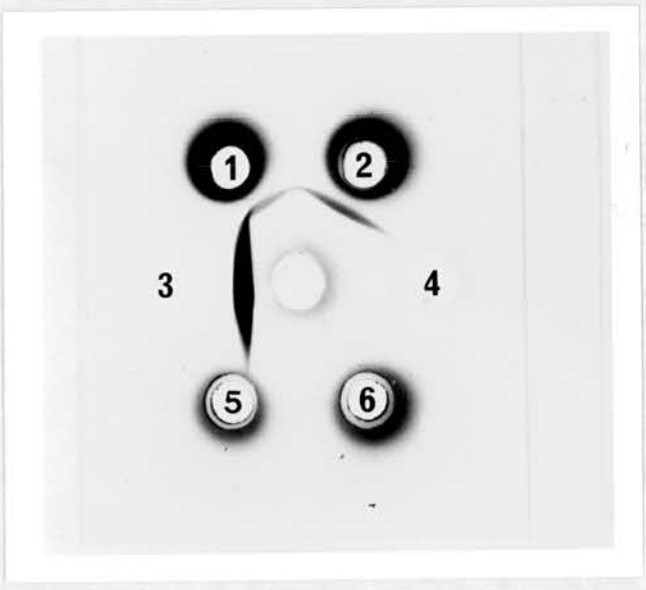
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concentrated by thimble dialysis to 1.3ml and to a final protein concentration of 0.25mg/ml. A total of 325ug IgE was purified from 5ml HIS. Assuming 100% recovery, the original concentration of IgE in HIS would therefore be 65ug/ml. The purity of the affinity purified IgE was checked by SDS-PAGE and Western blotting (see Chapter 3).

DOT-BLOTTING

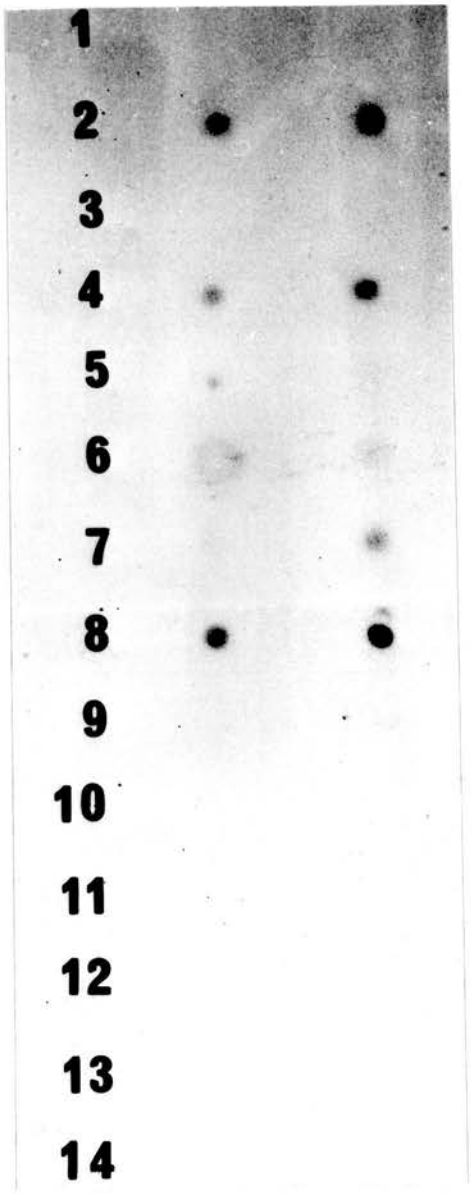
Eluates from the anti-IgE column were tested for presence of IgE by dot-blotting (Fig 2.4). Thirteen $1cm^2$ squares were marked contiguously on a dry sheet of nitrocellulose membrane. Into each was spotted 2ul neat serum or purified or partially purified IgE as shown in Fig. 2.4. The remaining protein binding sites were blocked by incubation in a PBS/egg albumin blocking buffer as described previously in the Western blotting procedure. The blot was probed with radio-iodinated monoclonal anti-rat IgE (Sera-lab, Sussex, U.K.) (diluted 1/1000) for 3 hours.

Crude myeloma protein and affinity-purified IgE from hyperimmune rat serum and myeloma were positive for IgE by dot-blotting (Fig 2.4). As little as 130 ng of pure IgE was detectable. However sera from rats collected 21 days after primary or 5 days after secondary infections with *N. brasiliensis* were negative on IgE blots (Fig. 2.4) as were normal rat serum and *N. brasiliensis*

Figure 2.4

Dot blots of affinity-purified IgE and other samples reacted with ^{125}I-labelled MARE 1. Columns A and B are duplicates, the non-reproducibility fo the results may be a consequence of the way the blotting paper was blanked.

1. HIS from F344 rats (day 7 after secondary infection).
2. IgE affinity-purified from F344 HIS (0.2mg/ml).
3. HIS from Lou rats (day 7 after secondary infection).
4. IgE affinity-purified from LOU HIS (0.2mg/ml).
5. IgE-depleted HIS from F344 rats (reconstituted to the original volume).
6. 50% ammonium sulphate precipitate of HIS from F344 strain.
7. IgE myeloma IR2, crude ascitic fluid (0.4 mg/ml).
8. IgE affinity-purified from myeloma IR2 ascites (0.13mg/ml).
9. Normal serum from Wistar rats.
10. *N. brasiliensis* AWH (0.3 mg/ml).
11. Single infection serum from F344 rats (day 17 after infection).
12. *N. brasiliensis* excreted/secreted antigens (0.1mg/ml).
13. IgE Affinity-purified from August HIS (0.1mg/ml).
14. Blank.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antigens. The absence of reactivity of immune serum may reflect the high plasma protein concentrations relative to that of IgE.

MEASUREMENT OF PARASITE-SPECIFIC IgE

The Passive Cutaneous Anaphylaxis (PCA) test was used to define anti-parasite IgE titres in sera from *N. brasiliensis*- infected rats. Following the method described by Ovary (1964), rats were sensitized by intradermal injection with serial two-fold dilutions of serum. 48 hours later, rats were given an intravenous injection of AWH (1000 worm equivalents) in a 1% Evans Blue (EB) solution. Following the same protocol, affinity-purified rat IgE was also tested by PCA.

One hour after i.v. injection of AWH and Evans blue, rats were killed by exsanguination from the carotid artery under deep anaesthesia and the dorsal skin removed to facilitate a quantitative assessment of the PCA reaction. The highest dilution of serum giving a blue spot greater than 5mm in diameter was taken as the titre.

CATABOLISM OF IMMUNOGLOBULIN BY RAT MAST CELL PROTEASES

Rat mast cell proteases I and II (RMCP) were purified by G. Newlands of the Moredun Research Institute, Edinburgh, according to the method of Gibson and Miller (1986). Specific enzyme activities were quantified by cleavage of a low MW substrate, CBZ-tyrosine-NPE and were expressed as nanokatals/milligram of

protein. 1 nanokatal is the activity required to hydrolyse 1nmol of substrate in one second (this procedure was performed by Dr. D. Knox of the MRI). Protein concentrations were calculated using the Pierce protein assay.

Monclonal rat IgG_{2a} antibody with specificity for horse radish peroxide (HRPO) was supplied in a purified form by Dr. S. Hobbs of the Institute of Cancer Research, Surrey. Polyclonal and monoclonal IgE were purified by affinity chromatography as described earlier in Materials and Methods.

FLOW CYTOMETRY

Operation of the FACScan

The instrument was operated as recommended by the manufacturer (Becton Dickinson, Oxford, U.K.). The machine was calibrated for the populations of cells to be analysed ensuring that Forward scatter (FSC), side scatter (SSC) and fluorescence (FL-1) fell within the analytical range of the instrument and were displayed on the screen in the form of histograms or dot plots. A "live gate" was set for each sample. This gate exluded dead cells from the analysis on the basis of very low FSC and SSC.

The machine was calibrated for analysis of peripheral blood leukocytes (PBL), bone marrow (BM) and peritoneal lavage cells. The parameter settings are shown in Table 1 and were stored on disc and recalled prior to analysis. Data were accumulated, stored and

analysed using "Consort 30" and "lysis" software packages (Becton Dickinson). Results were presented as dot-plots, with FSC on the Y-axis and SSC on the X-axis or by fluorescence channel number along the X-axis and number of cells on the Y-axis.

Table 1

Channel	Data Mode	Stage	Level
Forward Scatter	linear	Amplifier	1.00
		Detector	-
Side Scatter	linear	Amplifier	1.00
		Detector	321
Log Green Fluorescence	(Log)	Detector	637

Direct Fluorescent Antibody Technique

A direct fluorescent antibody technique was employed to demonstrate IgE on the surface of cells involving a single incubation step and only one immunological reagent per sample.

Cells were labelled with Fluorescein isothiocyanate (FITC)-conjugated murine monoclonal IgG_1 anti-rat IgE-FITC (MARE-FITC, Sera-Lab, Sussex, U.K.). VPM-FITC a monoclonal IgG_1-FITC, specific for a non-structural polypeptide of border disease virus was used as a negative control (supplied by Dr. Gary Entrican of the MRI). In a preliminary study, best results were noted when affinity purified MARE-FITC was used at a dilution of 1/200 (5 μg.ml). VPM-FITC was used at the same protein concentration (5 μg/ml. Cells were incubated with either conjugate in RPMI/1% BSA for 1 hour at $4^{o}C$ before washing x 3 in medium. Samples were fixed in 2% paraformaldehyde and stored at $4^{o}C$ in the dark until analysis by flow cytometry using FACScan.

CHAPTER 3

IDENTIFICATION OF ALLERGENS FROM ADULT *NIPPOSTRONGYLUS BRASILIENSIS* WORMS BY WESTERN BLOTTING

INTRODUCTION

The high incidence and severe clinical manifestations of allergic diseases in the human population have prompted detailed analysis of a number of clinically important allergens. Many helminth antigens provoke strong reaginic antibody responses in parasitized animals and may, therefore, also be classified as allergens. The relevance of parasite allergens to the host-parasite relationship has yet to be defined but there is sufficient epidemiological evidence in man (Hagan *et al.*, 1991) and experimental data from laboratory animals (Capron, Dessaint, Capron, Joseph and Pestel, 1980) to suggest that parasite allergens may induce host-protective IgE antibodies.

Before the advent of SDS-PAGE, molecular weights of proteins were estimated using gel exclusion chromatography. Similarly, parasite homogenates were separated by molecular sieving and fractions were tested for allergenicity by PCA (Wilson, 1967; Jones and Ogilvie, 1967). Unfortunately, the purification and characterization of nematode allergens is often limited by the low yields of parasite material and by their tendency to aggregate through protein-protein interactions (Ambler *et al.* 1972; Fujita, *et al.*, 1979; Owhashi, Horii, Ishii and Nawa, 1987). For example, the *A. suum* allergen, ASC-1, purified from AWH by gel-filtration had an apparent molecular weight of 30,000 to 40,000 (Hussain, Strejan and Campbell, 1972). Subsequent analysis by SDS-PAGE

demonstrated that ASC-1 is a dimer composed of two 8,200 MW polypeptides and that this allergen has a tendency to form homologous and heterologous aggregates in buffered solutions (Hussain *et al.*, 1973).

Similar results were obtained when J1, an allergen purified from *Schistosoma japonicum* eggs, had an apparent MW of 260,000 using gel filtration but using SDS-PAGE under reducing conditions the true MW was 135,000 (Owhashi *et al.*, 1987; Owhashii, Horii, Ishii and Nawa 1987b). The latter technique is a more reliable method of MW determination because it employs an anionic detergent (sodium-dodecyl-sulphate) and a strong reducing agent (mercaptoethanol) which minimize non-specific protein interactions and break down intra- and inter-chain disulphide bonding. However, the harshness of this technique changes the conformation of proteins and since allergenicity is dependant upon protein conformation (Ogilvie and Jones; 1967 Ambler and Orr, 1972), fractionation of putative allergens by SDS-PAGE may affect their allergenicity.

The earliest documented studies of *N. brasiliensis* allergens were conducted in the mid 1960's (Jones and Ogilvie, 1967; Wilson, 1967). Using gel filtration, Jones and Ogilvie (1967) partially purified a 12,000 - 17,000 MW protein from *N. brasiliensis* AWH. This protein was classified as an allergen because it induced PCA responses and caused systemic anaphylaxis after injection into rats immunized by previous infection. Ambler and Orr (1972) identified another allergen from *N. brasiliensis* AWH and, although they

failed to characterize the molecular weight of the allergen, its physicochemical properties were shown to be distinct from those of the 12,000 to 17,000 MW allergen identified by Jones and Ogilvie (1967).

The primary aims of the work described in this chapter were: 1) to identify the allergenic components in *N. brasiliensis* AWH using SDS-PAGE and Western blotting with IgE-specific probes and, 2) to assess whether affinity-purified IgE was more efficient in the detection of allergens than unfractionated serum IgE and 3) to assess whether an allergen thus identified and purified by electroelution retains biological activity using the PCA test.

RESULTS

Identification of *N. brasiliensis* Allergens by Western Blotting

Polypeptides from *N. brasiliensis* AWH antigens (500 μg protein) were separated by preparative SDS-PAGE (15%) and transferred to nitrocellulose by electroblotting. Blots were incubated with HIS, collected after secondary infection from Hooded Lister or LOU rats, and were subsequently probed with radiolabelled monoclonal anti-rat IgE antibody. Optimal detection of allergen was observed after diluting HIS 1/10 - 1/80 (Fig 3.1) but weak labelling was still demonstrable at dilutions of 1/320. LOU rats responded to six

Figure 3.1

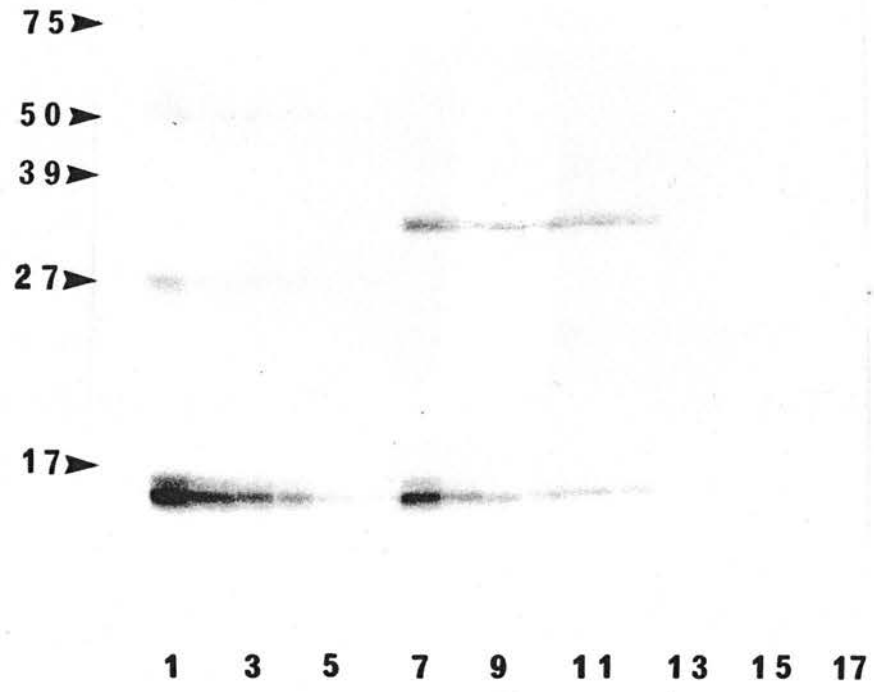
Detection of AWH allergens by SDS-PAGE and Western blotting. *N. brasiliensis* AWH (0.5mg) was fractionated under reducing conditions by SDS-PAGE (15%) and transferred to nitrocellulose membranes by electroblotting. Sera were collected from LOU and Hooded Lister rats infected with 3000 *N. brasiliensis* L_3 and subsequently reinfected with 5000 L_3. Nitrocellulose strips were probed with serial dilutions of pooled hyperimmune (HIS) and normal serum for 16 hours as listed below. Blots were subsequently incubated overnight with radiolabelled monoclonal mouse anti-rat IgE antibody (1/1000).

Key to Figure 3.1

Lane

1.	LOU HIS	1/10
2.	" "	1/20
3.	" "	1/40
4.	" "	1/80
5.	" "	1/160
6.	" "	1/320
7.	Hooded Lister HIS	1/10
8.	" "	1/20
9.	" "	1/40
10.	" "	1/80
11.	" "	1/160
12.	" "	1/320
13.	Hooded Lister normal	1/10
14.	" " "	1/20
15	" " "	1/40
16.	" " "	1/80
17	" " "	1/160

Molecular weight markers are indicated by arrows and are listed in Materials and Methods.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allergens with molecular weights of approximately 14,000-17,000, 28,000, 45,000, 50,000 and 69,000 and Hooded Lister rats recognized three allergens of 14,000, 17,000 and 32,000 MW. In both strains, the 14,000 MW allergen was most intensely labelled.

Analysis of the Allergenic Properties of Electroeluted Antigens

Four preparative gels were each loaded with 500 worm equivalents (w.e.) (642 μg protein) of *N. brasiliensis* AWH in reducing sample buffer. A central strip and the flanking regions of each gel were excised and stained with Coomassie blue. The region of the gel containing the 14,000 MW allergen was then identified and excised. Proteins were electroeluted and then concentrated before further analysis. Three faint bands with molecular weights of 14,000, 12,700 and 12,200 (Fig. 3.2), representing electroeluted proteins were demonstrable by SDS-PAGE. (Fig. 3.2). Although a 14,000 MW antigen was detected with parasite-specific IgE antibody on Western blots of AWH (Fig. 3.1), comparable labelling did not occur when the electroeluted antigens were re-analysed by SDS-PAGE, blotted and probed with HIS (results not shown). The electroeluted antigen was, nevertheless, tested for biological activity by PCA.

For PCA analysis, 3 rats were sensitized by intradermal injection with varying dilutions of HIS from Hooded Lister rats and 2 were challenged intravenously with 500 w.e. of electroeluted antigen (60 ug). Rat I was injected with 500 w.e. (642 μgs) of AWH. The latter provoked marked PCA reactions, demonstrable at a 1/200 dilution of HIS. Electroeluted antigens were less active by

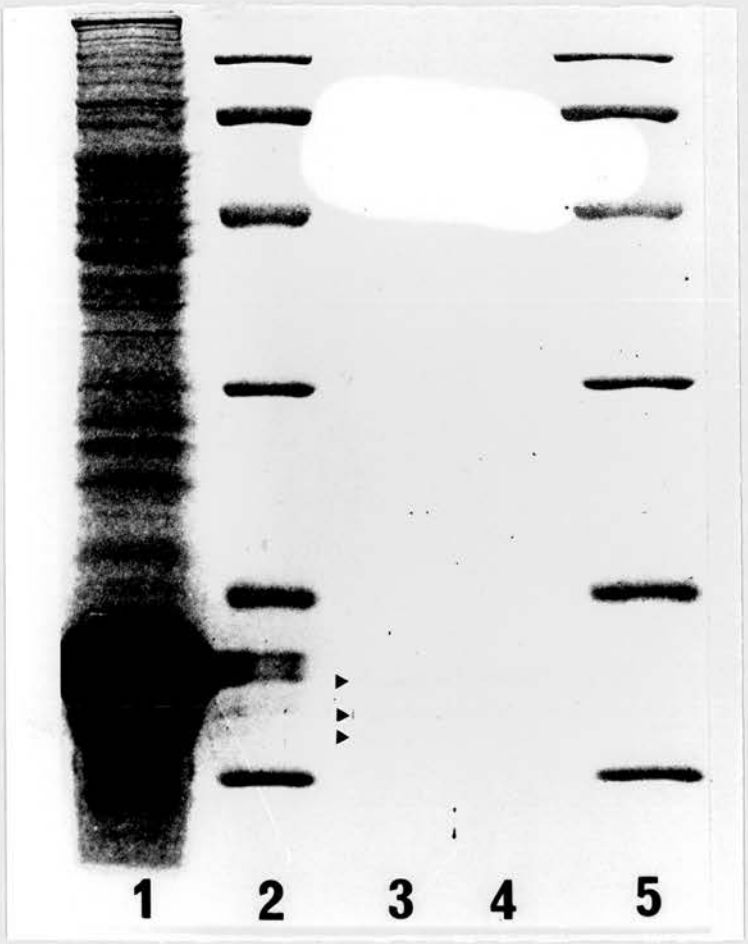
Figure 3.2

Electroeluted AWH proteins were analysed by SDS-PAGE (15%) under reducing conditions. The gel was subsequently stained with Coomassie blue.

Key to Figure 3.2

Lane

1. Untreated AWH (40μg).
2. Mol. Wts. x 10^3:-76, 66, 45, 25, 17 and 12 (10 μl).
3. Electroeluted allergen (5 μg).
4. Electroeluted allergen (5 μg).
5. Markers as shown in lane 5.



PCA, with an end point of 1/50 dilution of HIS in both rats II and III (Table 3-1). However rats II and III were challenged with 10% of the total protein used for rat I and it is likely that other allergens were present in the AWH.

Purification of IgE from HIS

Polyclonal IgE from LOU hyperimmune serum and monoclonal IgE derived from IR2 and IR162 myeloma ascitic fluids were affinity purified on a goat anti-rat IgE sepharose column. Preparations were examined by dot blotting (Fig. 2.4), SDS-PAGE, or Western blotting (Fig 3.3) and were tested for biological activity by PCA (Table 3-II, discussed later in the chapter).

Affinity-purified preparations of IgE, analysed by SDS-PAGE and stained with Coomassie blue, contained a major 66,000 MW polypeptide. Three additional proteins of 74,000, 49,000, and 23,000 MW were also present (Fig. 3.3). The 66,000 MW polypeptide was intensely labelled with ^{125}I-mouse monoclonal anti-rat IgE on Western blot. The 49,000 MW band was also labelled but the 23,000 MW polypeptide, only faintly detected with Coomassie blue stain, was unlabelled on Western blot (Fig. 3.3). The 66,000 MW polypeptides presumably represent epsilon heavy chain whereas the 49,000 MW polypeptide may be a degradation product and the unlabelled 23,000 MW protein is likely to be light chain.

Table 3-I

Analysis of electroeluted proteins by PCA in Wistar rats.

Dilution[a]	Diameter of reaction					
	I[b]		II		III	
NEAT	20[c]	22	16	14	13	14
1/10	14	16	12	10	11	9
1/50	8	9	2	1	4	3
1/100	5	4	-[d]	-	-	-
1/200	2	1	-	-	-	-
1/400	-	-	-	-	-	-
NRS[e]	-		-		-	
Saline	-		-		-	

Rat I was challenged with AWH (500 worm equivalents) and rats II and III were challenged with electroeluted antigen (500 worm equivalents). They were sensitized with HIS from Hooded Lister rats.

Key to Table 3-I

[a] Dilution of Hooded Lister HIS injected intradermally,
[b] Rats I-III were tested.
[c] Duplicate reactions measured in millimetres.
[d] No detectable PCA reaction.
[e] Normal rat serum.

Figure 3.3

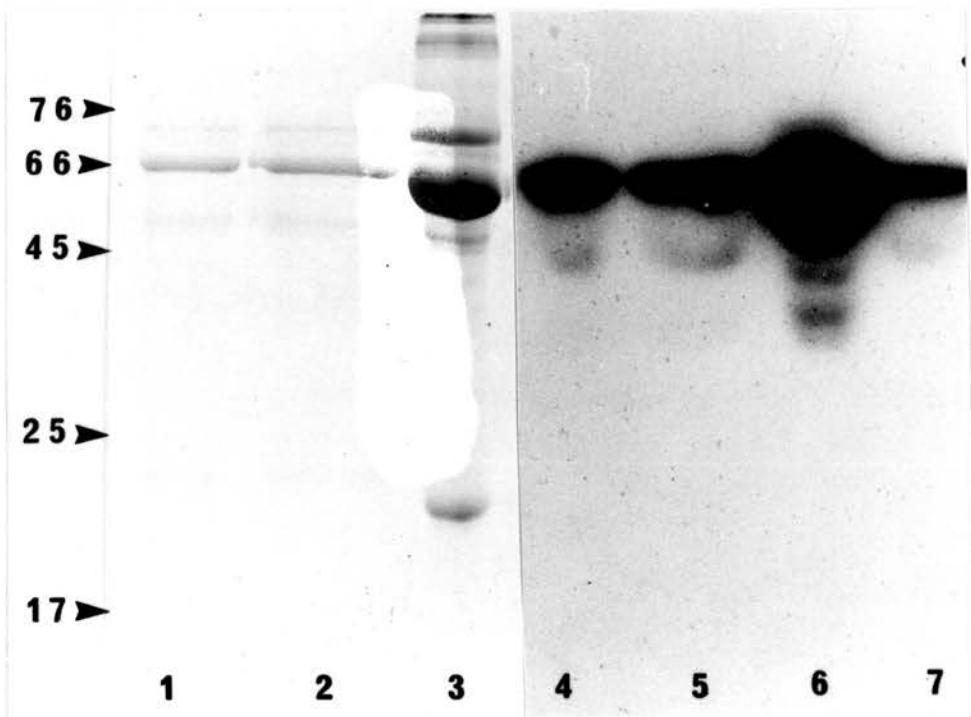
Analysis of purified polyclonal serum IgE and of myeloma IgE by SDS-PAGE and Western blotting. Crude and affinity-purified IgE preparations were fractionated by SDS-PAGE (12%) (Lanes 1 to 3) and stained with Coomassie blue or were blotted and probed with I^{125}-mAb anti IgE (1/1000) (lanes 4-7) overnight.

Key to Figure 3.3

Lane

1. Purified Serum IgE (1 μg)
2. Purified Myeloma IgE (1 μg)
3. Meyloma IgE IR2 Ascitic Fluid (5 μg)
4. Purified Serum IgE (1 μg)
5. " " IgE (2 μg
6. Myeloma IgE IR2 Ascitic Fluid (5 μg)
7. Purified Myeloma IgE (1 μg)

See Figure 3.1 for molecular weight markers



Specificity of Purified IgE

Proteins from *N. brasiliensis* AWH were separated by preparative SDS-PAGE and blotted as described in Materials and Methods. Blots were probed with IgE affinity-purified from HIS (pooled LOU sera), with IgE affinity-purified from IR2 myeloma IgE ascitic fluid, or with HIS from LOU rats previously infected several times with *N. brasiliensis*. Blots were probed with affinity-purified IgE within the range of concentrations described for serum from rats immunized by previous infection with *N. brasiliensis* (Jarrett *et al.*, 1976). Many AWH antigens including the 14,000 and 17,000 MW allergens were detected with IgE purified from HIS at a concentration of 58 μg/ml (Fig. 3.4; lanes 2, 3 and 4) whereas weak non-specific interactions with high molecular weight antigens occurred with purified myeloma IgE (Fig. 3.3; Lanes 11, 12 and 13) and the 14,000 and 17,000 MW allergens were not identified. The intensity of labelling was reduced at concentrations of 29 and 14.5 μg purified serum-derived IgE/ml but the 14,000 MW allergen was still clearly identified (Fig. 3.4). Blots of AWH incubated with monoclonal anti-rat IgE antibody remained unlabelled (Fig. 3.4; Lane 1).

Labelling of blotted worm antigen with purified myeloma IgE is likely to be non-specific, therefore in an attempt to reduce such interactions, blocking proteins were included at each incubation

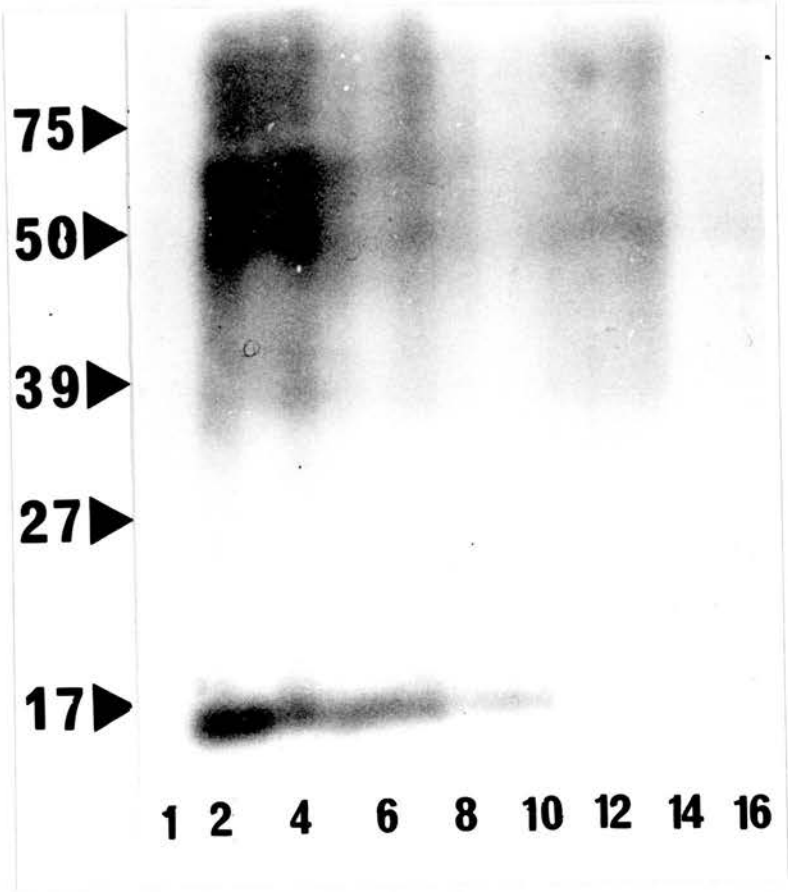
Figure 3.4

The specificity by Western blotting of myeloma IgE and IgE purified from HIS. AWH (0.5mg) was fractionated by SDS-PAGE (15%) and blotted. Blots were probed with 2ml of reagent, diluted in 5% Normal Rabbit Serum (RAB) or 5% egg albumin (Sigma Chemical Co.) as described below and subsequently with radio-iodinated anti-rat IgE (1/1000).

Key to Figure 3.4

Lane			
1.	Blank		
2.	IgE (HIS),	58μg/ml	-
3.	"	"	+ 5% RAB
4.	"	"	+ 5% Ovalbumin
5.	"	29μg/ml	-
6.	"	"	+ 5% RAB
7.	"	"	+ 5% Ovalbumin
8.	"	14.5μg/ml	-
9.	"	"	+ 5% RAB
10.	"	"	+ 5% Ovalbumin
11.	IgE (IR2)	58μg/ml	-
12.	"	"	+ 5% RAB
13.	"	"	+ 5% Ovalbumin
14.	"	14.5μg/ml	-
15.	"	"	+ 5% RAB
16.	"	"	+ 5% Ovalbumin

Molecular weight markers as in Figure 3.1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1 2 4 6 8 10 12 14 16

step as noted in the key to Fig. 3.3. Non-specific interactions were less pronounced where normal rabbit serum was added to IgE purified from HIS (Lane, 6, 9, 12 and 15) but not when ovalbumin was used (Lanes 4, 7, 10, 13 and 16).

In vivo Functional Analysis of Purified IgE

The capacities of IgE, affinity-purified from Hooded Lister HIS or from IR2 myeloma, and of untreated or IgE-depleted HIS to provoke PCA responses were compared (Table 3-II). Three Wistar rats were sensitized by intradermal injection with affinity-purified IgE. Rats II and III were challenged intravenously with 1000 w.e. of AWH in saline/1% Evan's blue, 24 and 48 hours respectively, after intradermal sensitization. In order to test for the presence of aggregated or complexed IgE in affinity-purified preparations, an intravenous injection of 1 ml 1% Evan's blue (EB) in saline was given to rat I an hour after intradermal sensitization. For control purposes, each rat was also sensitized intradermally with HIS, IgE-depleted HIS, and normal rat serum (Table 3-II).

The PCA titres of purified immune IgE were similar regardless of whether they were assessed 24 (rat II) or 48 hours (rat III) after sensitization. Strong PCA reactions were noted with 12.5 μg of affinity-purified IgE from Hooded Lister HIS and reactions were

Table 3-II

Analysis of Purified IgE for biological activity and specificity by the PCA reaction.

Sensitizing Reagent	I[a]		II		III	
Specific-IgE[b]	[c]					
12.5	8	7	17	20	16	14
6.25	4	2	10	14	15	14
3.125	3	2	8	12	7	7
1.56	2	2	4	6	4	3
0.78	1	1	3	-[g]	3	-
0.39	1	1	-	-	-	-
0.195	1	1	-	-	-	-
CONTROLS						
MYELOMA IgE 12.5[b]	4		-		-	
HIS[d]	1		22		24	
IgE-HIS[e]	1		-		-	
NRS[g] neat	1		-		-	
SALINE	1		-		-	

Rat I was given EB/saline only, intravenously, 1 hour after dermal sensitization and rats II and III were challenged with 1000 w.e. in EB/saline, 24 and 48 hours respectively after sensitization.

Key to Table 3-II

[a] Rats I - III were tested.
[b] quantity of IgE (μg) used for intradermal sensitization
[c] duplicates
[d] Hooded Lister HIS
[e] IgE depleted HIS (neat)
[f] normal rat serum (neat)
[g] no reaction

still evident with 1.56 μg (Table 3-II). Purified Myeloma IgE, IgE depleted HIS, NRS, and saline all failed to produce PCA reactions. By contrast, undiluted Hooded Lister HIS provoked a strong response (Table 3-II).

Local IgE-induced blueing was noted in rat I given Evan's blue 1 hour after intradermal sensitization. IgE purified from HIS induced concentration-dependant dermal reactions and, similarly, myeloma IgE caused a local, but less intense blueing. In contrast, HIS, IgE-depleted HIS, and normal rat serum were without effect and skin reactions were similar to those of the saline controls (Table 3-II).

Serum Immunoglobulin Isotype Responses to AWH

Sera from uninfected and from hyperimmune Hooded Lister rats, collected 1 week after tertiary infection, were screened for parasite-specific antibodies by Western blotting. Parasite-specific antibody isotypes other than IgE were detected with polyclonal sheep antibodies against IgG_1, IgG_{2a}, IgG_{2b}, IgG_{2c} and IgA, followed by pig anti-sheep IgG-peroxidase conjugate. Analysis of blotted whole worm antigens probed with HIS followed by polyclonal rabbit anti-rat IgG revealed many bands in the molecular weight range of > 200,000-30,000. The results were very similar with the isotype-specific antibodies. Polypeptides of 73,000 and 50,000 MW were detected with normal rat serum (Fig. 3.5; Lanes 9 to 13) particularly when probed with sheep anti-IgG_{2c} and anti-IgA (lanes 12 and 13). Bands in the > 200,000-30,000 MW range were faintly

Figure 3.5

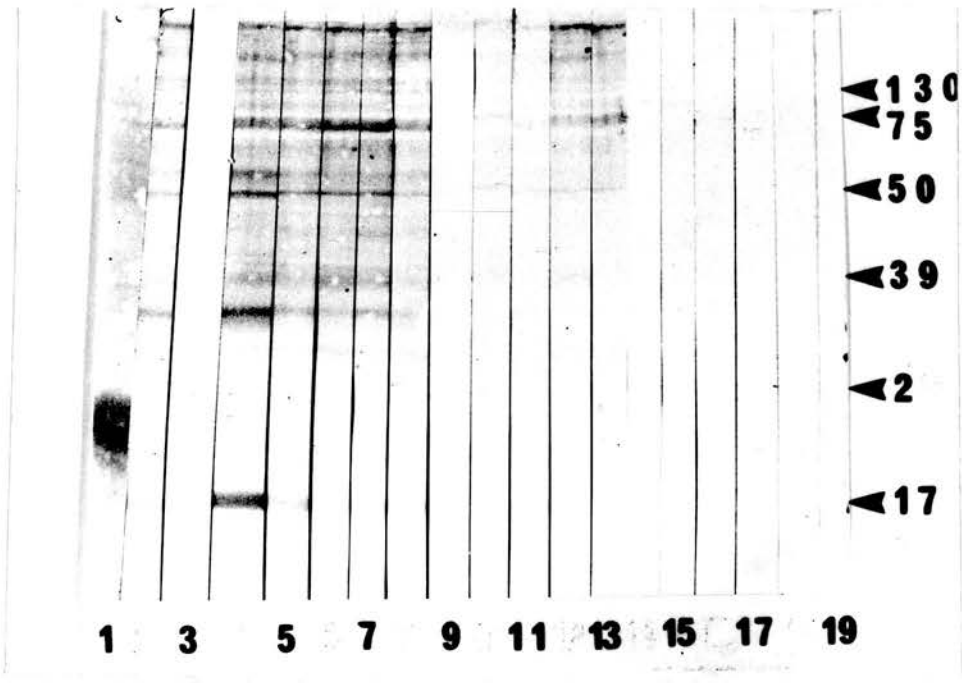
Specificities of Hooded Lister hyperimmune and normal serum antibodies defined by Western blotting. *N. brasiliensis* AWH (0.5mg) proteins were fractionated by SDS-PAGE (15%) and blotted. Blots were first probed for 1 hour with hyperimmune (HIS) or normal rat sera (NRS) (diluted 1/1000) and subsequently probed for 2 hours with sheep anti-rat isotype specific sera, (diluted 1/200) and peroxidase conjugate (1/200 for 2 hours), as listed below.

Key to Figure 3.5

Lane

1. Blotted AWH antigen stained with Coomassie blue stain.

	Serum	Isotype	Peroxidase Conjugate
2.	HIS	BLANK	Rabbit anti-rat IgG
3.	NRS	BLANK	" " "
4.	HIS	IgG1	Pig-anti-sheep IgG
5.	"	IgG2a	" " "
6.	"	IgG2b	" " "
7.	"	IgG2c	" " "
8	"	IgA	" " "
9.	NRS	IgG1	" " "
10.	"	IgG2a	" " "
11.	"	IgG2b	" " "
12.	"	IgG2c	" " "
13.	"	IgA	" " "
14.	BLANK	IgG1	" " "
15.	"	IgG2a	" " "
16.	"	IgG2b	" " "
17.	"	IgG2c	" " "
18.	"	IgA	" " "
19	Molecular weight markers		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labelled with normal rat serum (Fig. 3.5). By contrast, an antigen of approximately 17,000 MW and specifically detected with HIS and the most intense labelling was obtained with sheep anti-rat IgG_1 (Fig. 3.5). The apparent affinity of IgG_1 antibody for the 17,000 MW antigen, suggests that there may be competition between antibody isotypes for the same epitopes. This is especially relevant for the analysis of IgE, because an antigen of the same molecular weight (see Chapter 4) binds IgE antibody on immunoblots. Polyclonal anti-rat IgG and IgG_{2a} also detected this antigen but labelling with other isotype-specific antibodies was much weaker.

Competition between serum IgG and IgE Antibody for Allergen Epitopes

The binding of parasite-specific IgG_1 to worm antigens on Western blots, and particularly to the 17,000 MW antigen, suggested that this isotype could competitively block the binding of IgE to worm allergens. Such a possibility was assessed by treating electroblots of AWH with IgE-depleted HIS from Hooded Lister rats, or with normal rat serum thus permitting antibodies other than the IgE isotype to bind and perhaps block AWH epitopes. Blots were subsequently probed with IgE which had been affinity purified from Hooded Lister HIS. The binding of IgE and any inhibition of binding were identified by a final incubation with radiolabelled monoclonal anti-rat IgE (Fig. 3.6). For control purposes, individual nitrocellulose strips were incubated in the absence of rat serum (blank), or with affinity purified myeloma IgE, before incubation with radiolabelled monoclonal anti-rat IgE (Fig. 3.6).

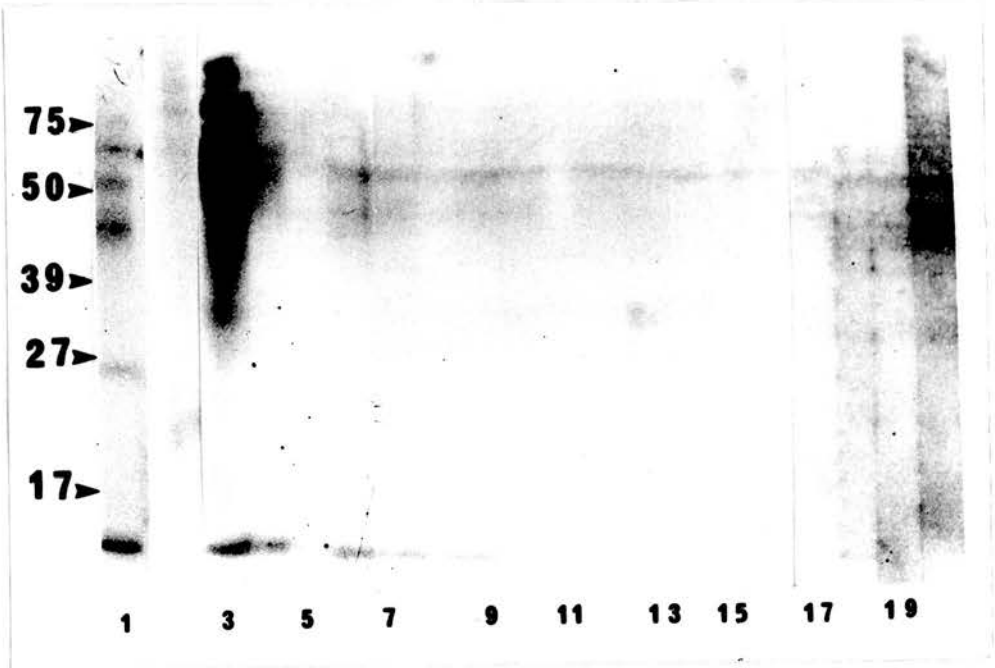
In the high molecular weight range there was apparent non-specific binding of IgE to blots of AWH (Fig. 3.6). Nevertheless the 14,000 MW allergen was clearly identified in lane 3 incubated with purified IgE (40ug/ml) in the absence of prior treatment with IgE-depleted HIS. Labelling of the 14,000 MW allergen was reduced or absent in lanes 4 and 5, pre-treated, respectively with 1 or 20% IgE-depleted HIS. A similar pattern to that in lane 3 was observed in lane 6 treated with IgE (20ug/ml) alone where the 14,000 MW allergen was clearly identified, whereas, in lanes 7 and 8, following pretreatment with IgE-depleted HIS, labelling of the 14,000 MW allergen was weak or undetectable. In blots (lanes 14-20) treated uniquely with IgE-depleted HIS or with IgE myeloma the 14,000 MW allergen was not identified.

Figure 3.6

Western blot showing the specific and non-specific binding of IgE, purified from HIS or IR2 ascitic fluid, to worm antigens. *N. brasiliensis* AWH (0.5mg) was fractionated by SDS-PAGE (15%) and blotted. Blots were probed with the primary (1 hour) and secondary (16 hours) step reagents as listed below. Blots were then incubated in radio-labelled monoclonal anti-rat IgE (1/1000) for 16 hours.

Key to Figure 3.6

Lane	S T E P 1	S T E P 2
1.	HIS 1/20	B L A N K
2.	NRS 1/20	" "
3.	NRS 1/100	IgE (HIS) 40μg/ml
4.	IgE depleted HIS 1/100	" "
5.	" " " 1/5	" "
6.	NRS 1/100	" 20μg/ml
7.	IgE depleted HIS 1/100	" "
8.	" " " 1/5	" "
9.	NRS 1/100	" 10μg/ml
10.	IgE depleted HIS 1/100	" "
11.	" " " 1/20	" "
12.	" " " 1/10	" "
13.	" " " 1/5	" "
14.	" " " 1/100	B L A N K
15.	" " " 1/20	" "
16.	" " " 1/5	" "
17.	NRS 1/100	" "
18.	" "	IgE (IR2) 10μg/ml
19.	" "	" 20μg/ml
20.	" "	" 40μg/ml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DISCUSSION

This chapter describes the fractionation of *N. brasiliensis* AWH by SDS-PAGE and the characterization of putative adult worm allergens by Western blotting. Worm homogenates were solubilized in PBS to permit comparisons with previous analyses of allergens from *N. brasiliensis* (Wilson, 1967; Jones and Ogilvie, 1967; Ambler and Orr, 1972; Wedrychowicz *et al* 1986). Blotted AWH antigens to which immune IgE binds are assumed to be allergens although, under reducing conditions, it is possible that some conformational epitopes were not detected.

Earlier work identifying allergens by SDS-PAGE and Western blotting with atopic human sera and anti-IgE includes that of Sutton, Wrigley and Baldo, (1982), who identified the allergenic components in a complex cereal grain mixture and of Duffort, Carreira and Lombardero, (1987) who characterized the cat dander allergens. Tovey and Baldo, (1985) and Bengtsson, Rolfsen and Einasson, (1987) have, similarly, employed these techniques to identify pollen and house dust mite allergens respectively.

In this study, seven distinct allergens of 14,000, 17,000, 28,000, 32,000, 45,000, 50,000 and 69,000 daltons were identified in AWH by Western blotting. The 14,000 MW allergen may be similar to the 12,000 to 17,000 MW allergen partially purified by Jones and Ogilvie (1972) and the 12,600 MW allergen described by Wedrychowicz *et al.* (1986). Comparisons have been made between this allergen and a secreted allergen of a similar size. Wilson (1967) and more

recently Petit, *et al* (1980) identified an allergen with a molecular weight of 12,000 to 15,000 in excreted and secreted (ES) products. Both allergens were active by PCA, possessed similar physicochemical properties (Petit *et al.*, 1980; Wedrychowicz *et al.*, 1986), and induced comparable anaphylactic reactions in the skin. Therefore these different observations may reflect a single allergen where small variations in molecular weights may be a consequence of different processing mechanisms required for somatic and secreted proteins. By using IgE blotting, the present results show two distinct allergens of 14,000 and 17,000 MW, a distinction which was not possible using column chromatography and PCA reactions (Petit *et al.*, 1980; Wedrychowicz *et al.*, 1986).

The 50,000 MW allergen identified in this chapter may be similar to the allergen of the same size purified from ES (Wedrychowicz *et al.*, 1986). It is not uncommon for secreted allergens to be present in parasite homogenates (Ambler *et al.*, 1973; Fujita and Tsukidate, 1982; Yamada *et al.*, 1991). Although Ambler and Orr, (1972) and McWilliam *et al.* (1986) failed to characterize the molecular weights of their allergens, it is possible that the same allergens were identified in the present experiment.

A recently published study using Western blotting and SDS-PAGE under non-reducing conditions has described a number of allergens in ES and AWH from *N. brasiliensis* (Yamada *et al.* 1991). Six allergens of 130,000, 70,000, 58,000, 48,000, 28,000 and 24,000 MW were detected with sera collected from Sprague Dawley rats. Some of the allergens described by Yamada *et al.* (1991) are of similar

MW to those described here. For example allergens of 28,000, 45,000- 48,000, and 69,000 - 70,000 MW. They did not, however, identify the low MW allergens (Yamada *et al.* 1991) described in this chapter. The reason for this discrepancy is not clear although it is possible that, under non-reducing conditions there was some protein-protein interaction and the 14,000 MW allergen was not resolved.

The 14,000 MW polypeptide and two other proteins of similar molecular weight were electroeluted and, together, proved to be allergenic by PCA. This result, although not conclusive, suggests that Western blotting does indeed detect allergens at least some of which, even after electrophoresis, remain allergenic. Alternatively, the allergens renature sufficiently after electroelution to remain biologically active.

Allergens can stimulate the production of IgG antibody (Ottesen, Kumaraswan, Paranjapa, Poindexter and Tripathy, 1981; Ito *et al.*, 1986; Kemeny and Lessof, 1987) which may compete with, and thereby block IgE binding. Blocking antibody has been described in previous studies of infection with *N. brasiliensis* (Jones and Ogilvie, 1967) where AWH lost allergenicity after treatment with immune serum. This was thought to be a consequence of specific IgG antibodies blocking the binding of IgE to allergens. Blocking IgG antibody has also been described previously in human filariasis (Ottesen *et al.*, 1981). Immunizing regimes to engender competition between IgG and IgE isotypes have been used therapeutically by

clinicians to minimize allergic reactions in man (Yamumuchi, Ito, Ishii and Miyamoto, 1986) and against some seasonal allergies (Moss, Hsu, Kwanicki, Sullivan and Reid, 1987; Deards, Standrung and Moran, 1986).

To be confident of identifying all allergens in *N. brasiliensis* AWH, it was considered necessary to purify IgE from HIS. It was also hoped that this novel approach might enhance the early detection of parasite-specific IgE. Western blots were probed with IgE at concentrations of 14.5, 29 and 58 μg/ml. These concentrations were chosen because they are within the range of total concentrations of IgE in rat serum during nematode infections (Jarrett *et al.*, 1976).

When used to probe Western blots, purified IgE bound a number of high molecular weight parasite antigens in an apparently non-specific manner. This was particularly obvious when using high concentrations of IgE and was reduced only slightly when blots were incubated with blocking proteins before incubation with IgE. The 28,000, 45,000, 50,000 and 71,000 MW allergens, identified by blotting with HIS, could not be distinguished from background labelling when blots were developed with IgE purified from the same HIS. The usefulness of purified IgE as a blotting reagent is, therefore, limited. On the other hand the low molecular weight allergens were detected in a clear and specific manner by purified IgE. The acid elution of IgE from the affinity column may have had a denaturing effect, thereby reducing its specificity on blots although purified IgE retained some of its biological activity.

Similarly, after intradermal injection of IgE, reactions were observed in a control rat given intravenous saline and Evans blue one hour later. This observation suggested that either IgE aggregates or IgE/antigen complexes might be present. Whatever the cause of this phenomenon the potential for inducing substantial PCA reactions following intravenous challenge with allergens is probably reduced.

In order to assess the potential blocking of the binding of IgE to allergens by other antibody isotypes, the specificities of other immunoglobulin isotypes were examined, and their binding to AWH antigens evaluated. All antibody isotypes from HIS had varying degrees of parasite specificity and the IgG_1 subclass had apparently the most comprehensive repertoire, recognizing many parasite antigens and especially a 14,000 MW antigen, a putative allergen.

When blots of AWH were pre-treated with HIS depleted of IgE, and subsequently probed with purified immune IgE, this regime reduced the intensity of labelling of the 14,000 and 17,000 MW allergens, as well as the high molecular weight antigens, whereas normal rat serum was without effect. These observations indicate the presence of blocking antibodies, possibly of the IgG_1 and IgA isotypes. However, blocking antibody did not completely inhibit the binding of IgE to allergen and it is possible that IgG and IgE bind to distinct epitopes, or that IgE has a stronger affinity than IgG.

Although the ability of normal rat serum to inhibit IgE antibody was not tested, it was apparent from Western blotting that normal rat serum does contain some specificity for blotted AWH. Similar results were observed with ELISA and this binding could be partially inhibited by blocking with phosphorylcholine (results not shown). McWilliam *et al.*, (1986) and Mitchell *et al* (1976) also noted that *N. brasiliensis* antigens expressed phosphorylcholine haptens. Similar observations have been reported for *T. spiralis* (Ubeira, Leiro, Seone and Regueiro, 1987) and *A. suum* (Gutman and Mitchell, 1977). Therefore it is likely that the reaction of normal rat serum with *N. brasiliensis* antigen is associated with antibodies to common epitopes such as phosphorylcholine.

In conclusion, the Western blotting technique was successfully employed to detect *N. brasiliensis* AWH allergens. Seven allergens were identified when AWH blots were probed with HIS. The 14,000 MW allergen was electroeluted from the polyacrylamide gel, and although the sample contained 2 additional polypeptides, importantly, this low MW (presumably 14,000 MW) allergen remained biologically active after SDS treatment and electroelution. Purified immune IgE was bioactive by PCA and relatively free of contaminants as shown by SDS-PAGE and Western blotting. Unfortunately purified IgE bound non-specifically to proteins of high molecular weight. AWH electroblots were first treated with

IgE-depleted HIS, and then with purified immune IgE. It was clear from this analysis that IgE binding was reduced, suggesting that direct competition for epitopes or steric hindrance occur when sequential blotting is performed in this manner.

CHAPTER 4

COMPARISON OF IgE RESPONSES TO INFECTION WITH *N. BRASILIENSIS* BY DIFFERENT STRAINS OF RAT

Introduction

Histological and biochemical studies have shown that numbers of intestinal mast cells increase dramatically during enteric nematode infection (Miller and Jarrett, 1971; Woodbury and Miller, 1982). Furthermore, intestinal mast cells are activated during parasite expulsion as determined by the systemic secretion of soluble granule proteases into blood during infection (Miller *et al.*, 1983; Woodbury *et al.*, 1984; Huntley *et al.*, 1990). The strong functional link between mast cells and IgE and the well-known association between nematodiasis and reaginic antibodies (Ogilvie, 1967) have prompted many detailed analyses of parasite-mediated hypersensitivity reactions, and there have been numerous studies of the kinetics of parasite-specific IgE production using the PCA test (reviewed in Jarrett and Miller, 1982).

Although not detected in serum by PCA before the commencement of worm expulsion in rats (Ogilvie, 1967; Wilson and Bloch, 1968; Keller, 1970; Jarrett *et al.*, 1976; Befus *et al.*, 1982), *N. brasiliensis*-specific homocytotropic antibody was demonstrable on peritoneal lavage cells and within mediastinal (bronchial) lymph nodes 5 and 10 days, respectively, prior to its detection in serum (Wilson and Bloch, 1967; Befus, *et al.*, 1982). Synthesis of parasite-specific IgE may, therefore, precede the detection of anaphylactic antibodies in serum by several days (Urquhart, 1965; Wilson and Bloch, 1967; Befus *et al.*, 1982).

The primary aims of the experiments in this chapter were 1) to analyse and compare the specificity and time-course of IgE responses to *N. brasiliensis* in different rat strains using Western blotting (Experiment I) and, 2) to confirm that the serum IgE responses were comparable to those described by other workers (Jarrett *et al.*, 1976; Befus *et al.*, 1982), using the PCA test (Experiment II).

Experiment I: Primary secondary and tertiary IgE responses in 4 strains of rats infected with *N. brasiliensis*, analysed by Western blotting

Protocol. Ten rats from each of the four strains, LOU, F344, Hooded Lister and August, were infected with 2000 *N. brasiliensis* L3. Animals were bled, under anaesthesia, from the tail vein immediately before infection (day 0), and 7, 10, 14, and 21 days after infection (DAI). Faecal egg counts were monitored and 3 rats from each strain were killed on day 7 for worm counts (Table 4-I)

In order to further boost IgE responses, rats from F344 and LOU strains were given a secondary infection with 3000 L_3 on day 52 and a tertiary infection with 5000 L_3 17 days later. They were bled 3, 7 and 14 days after secondary infection and 7 days after tertiary infection (day 21). All sera were examined for parasite-specific IgE by Western blotting although the volumes of serum obtained by tail bleeding were insufficient to permit further analysis using PCA.

Table 4-I

Worm burdens in different strains of rat.

	F344	LOU	August	Hooded Lister
	1509	1421	1387	1527
	1583	1480	1672	1675
	1624	1469	1342	1304
Mean	1572	1467	1467	1503
± SD	58	79	79	187

Rats were killed 7 days after primary infection with 2,000 *N. brasiliensis* L_3

For SDS-PAGE analysis, the gels, comprising a 4% stacking gel and a 15% homogeneous resolving gel, were loaded with 200µl *N. brasiliensis* AWH, diluted in reducing sample buffer to a final concentration of 2.5 mg/ml. After electrophoresis, worm antigens were electroblotted onto nitrocellulose which was cut into strips and each was incubated overnight with serum samples from individual rats and subsequently with [^{125}I]-monoclonal anti-rat IgE (1/1000 dilution) for 16 hours (see Materials and Methods)

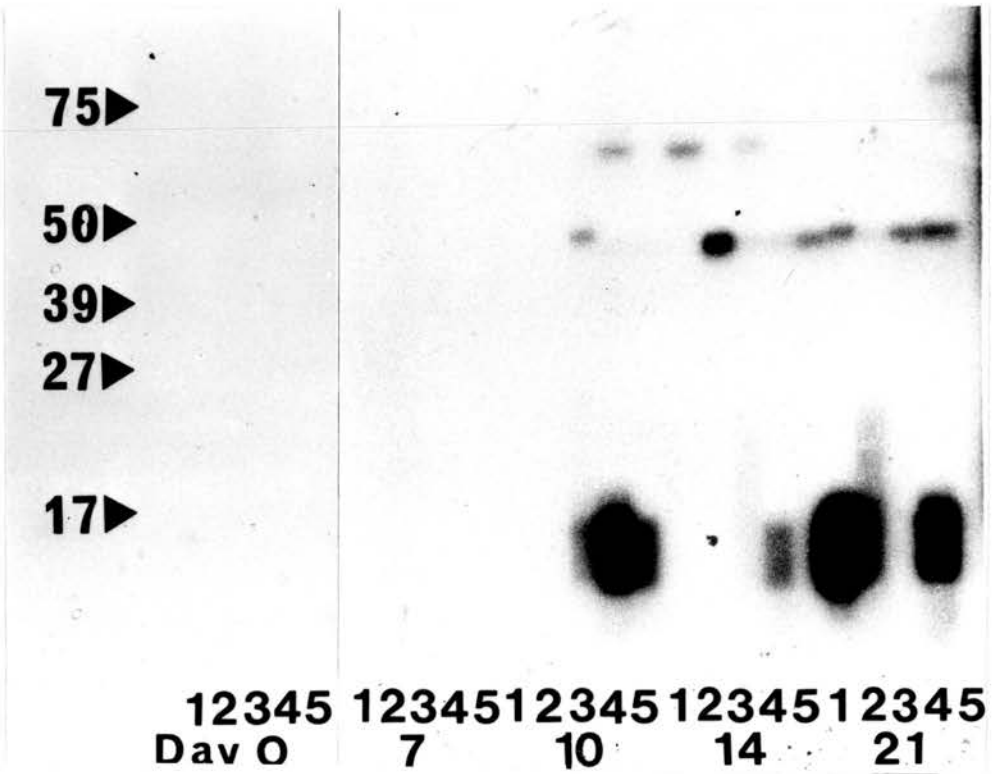
RESULTS

(i) Primary IgE response to infection with *N. brasiliensis*

(a) LOU. Parasite-specific IgE was not detected in serum from Lou rats by Western blotting on days 0 and 7 (Fig. 4-1). By day 10 three rats (3, 4 and 5) responded to allergens of 14,000 and 17,000 MW (Fig. 4-1). However, these precise MWs were only

Figure 4-1

Western blots showing primary IgE responses of adult LOU rats to infection with *N. brasiliensis*. Serum samples were collected from 5 rats 0, 7, 10, 14 and 21 days after primary infection. *N. brasiliensis* AWH (0.5 mg) was fractionated by SDS-PAGE (15%) under reducing conditions. After blotting, separate strips were probed for 16 hours with individual serum samples diluted 1/20 and then with radio-iodinated anti-rat-IgE (1/1000) for a similar time period. This blot was over-exposed to ensure maximal detection of parasite-specific IgE directed against high MW allergens. Molecular weight markers (see Figure 3-1 for key) are arrowed on the left and the groups (1-5) of individual rats and days after infection on which they were bled are shown below.


75▶
50▶
39▶
27▶
17▶
12345 12345 12345 12345 12345
Dav O 7 10 14 21

demonstrable on blots which were relatively underexposed (not shown). Two of the three (3 and 4) also responded to 50,000 and 69,000 MW allergens (Fig. 4-1) whereas rats 1 and 2 remained unresponsive (Fig. 4-1). Only one animal (rat 4) was still producing IgE specific for the low molecular weight allergens on day 14. However, IgE antibodies against the high molecular weight allergens (50,000 and 69,000 MW) were present in sera from all five animals (Fig. 4-1). By day 21, rats 1, 2 and 4 responded very well to 14,000, 17,000, and 50,000 MW allergens and rats 3 and 5 responded uniquely to 50,000 and 76,000 MW allergens, respectively.

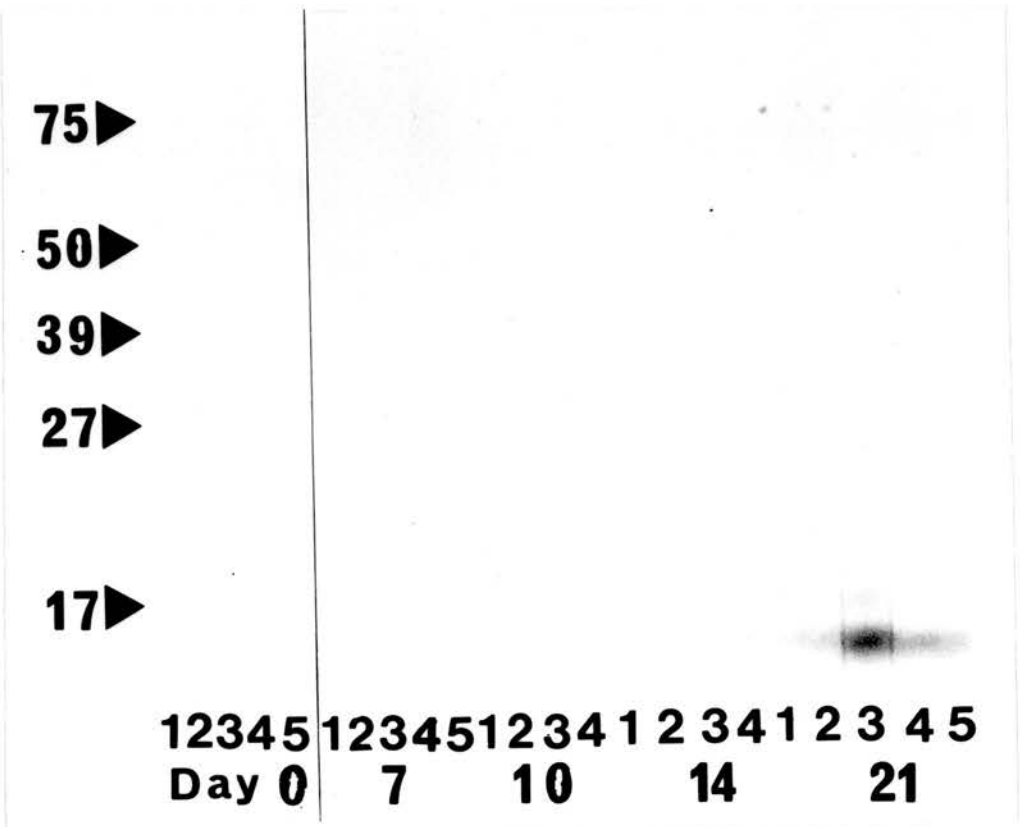
b)F344. Repeated Western blotting failed to reveal an IgE response by F344 rats at any time after primary infection despite prolonged exposure of blots (data not shown).

c) August. Parasite-specific IgE in serum from August rats was first detected by Western blotting, on day 21 (Figure 4-2). All five rats had antibodies against a 14,000 MW allergen and rats 1 and 3 also responded to a 17,000 MW allergen. The intensity of these responses was variable with weak reactions against the 17,000 MW allergen. All five rats were unresponsive to the higher MW allergens against which LOU rats had responded (Figures 3-1 and 4-1).

d) Hooded Lister. IgE specific for 14,000 and 17,000 MW allergens was detected on day 7 and at all time points (days 10, 14, and 21) thereafter, with very little variation between rats.

Figure 4-2

Western blots demonstrating primary IgE responses by August rats against *N. brasiliensis*. Conditions and labelling are described in Figure 4-1.



The responses on days 7 and 14 were, however, rather weak and did not reproduce well (Fig. 4-3). The specificity of IgE from Hooded Lister rats, like that of the August strain, was selective for 14,000 and 17,000 MW allergens.

Faecal Egg counts. Pooled faecal samples were collected daily from each group of rats, and faecal egg outputs were calculated and expressed per gram of faeces for each strain (Table 4-II). Total output varied between strains but kinetics were comparable with egg counts reaching a peak between days 6 and 9 and falling to near zero by day 12.

(ii) IgE responses during secondary and tertiary infections with *N. brasiliensis*

a) LOU. Three days after secondary infection there were IgE antibodies against two low molecular weight allergens (Fig. 4-4). Because the 17,000 molecular weight markers did not migrate the full distance into the gel, the MWs of these allergens were not determined, although it is possible that they are the same 14,000 and 17,000 allergens recognized after primary infection and subsequent SDS-PAGE analysis (not shown) has confirmed this. A single rat (No. 5) produced a weak IgE response against the smaller of the two allergens (presumably 14,000 MW) on day 3 (Fig. 4-4). The IgE response increased in intensity with time after secondary and tertiary challenge infections and, in 4 of the 5

Figure 4-3

Primary IgE responses by Hooded Lister rats to *N. brasiliensis* are demonstrated by Western blotting. See Figure 4-1 for conditions used and for the key to labelling. Serum from normal Hooded Lister (A) and LOU (B) rats were included as controls (1/20).

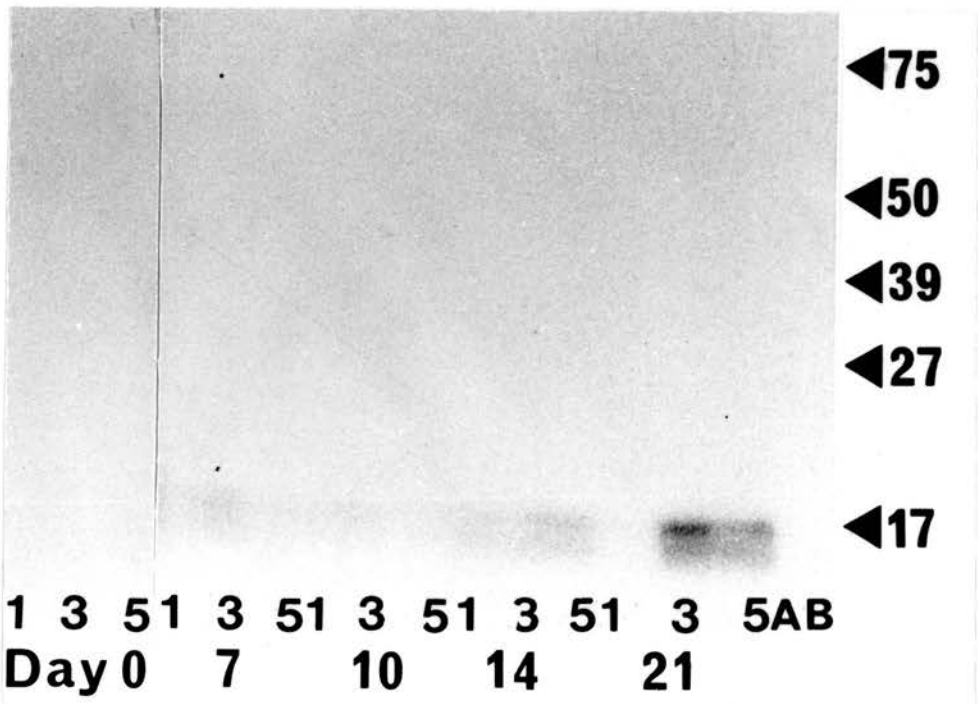


Figure 4-4

Secondary and tertiary IgE responses by LOU rats to adult *N. brasiliensis* worm antigens are analysed by Western blotting. Samples were collected 3, 7 and 14 days after secondary challenge and rats were given a third infection on day 14. The final samples, taken 7 days after the third infection (day 21) were also analysed. The techniques involved and the key to the figure are described in more detail in Figure 4-1. Sera from normal LOU (A) and F344 (B) rats were included for control purposes.

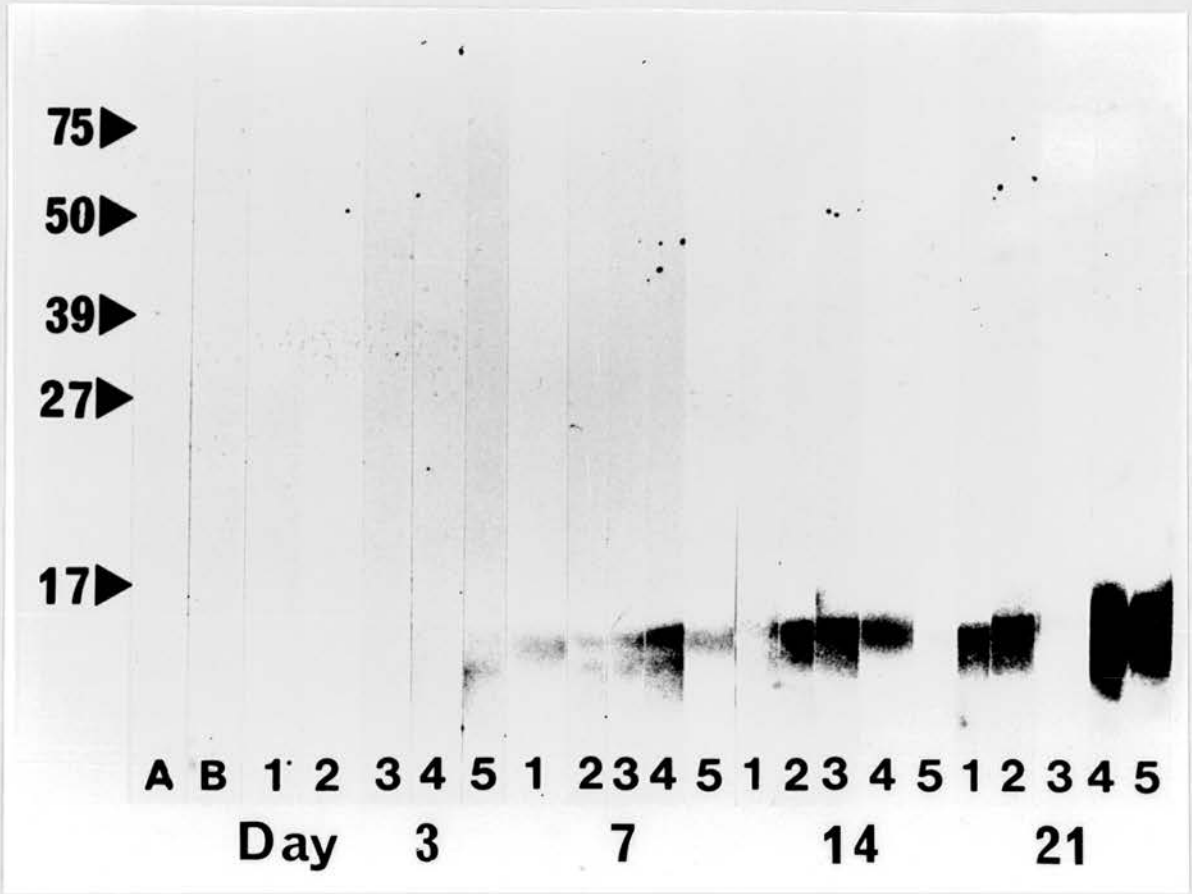


Table 4.II

Faecal egg counts in four strains of rat infected with 2,500 *N. brasiliensis* larvae

Time	Strain			
	F344	LOU	Hooded Lister	August
3+	0*	0	0	0
4	12	10	12	114
5	319	432	1,908	35
6	3,850	1,750	44,860	2,622
7	14,612	9,801	4,367	9,832
8	30,275	19,872	963	23,349
9	40,956	35,787	1,012	7,651
10	15,642	22,361	20	15
11	4,232	5,937	0	19
12	10	52	0	0
13	-	-	-	-
14	-	-	-	-

+ time in days
* pooled faecal egg counts/gram of faeces

rats, the strongest responses were noted on day 21 (7 days after tertiary challenge). Serum IgE specific for allergens of 50,000, 69,000 and 67,000 MW, evident during primary infection (Fig. 4-1), was absent after secondary and tertiary infections (Fig. 4-4).

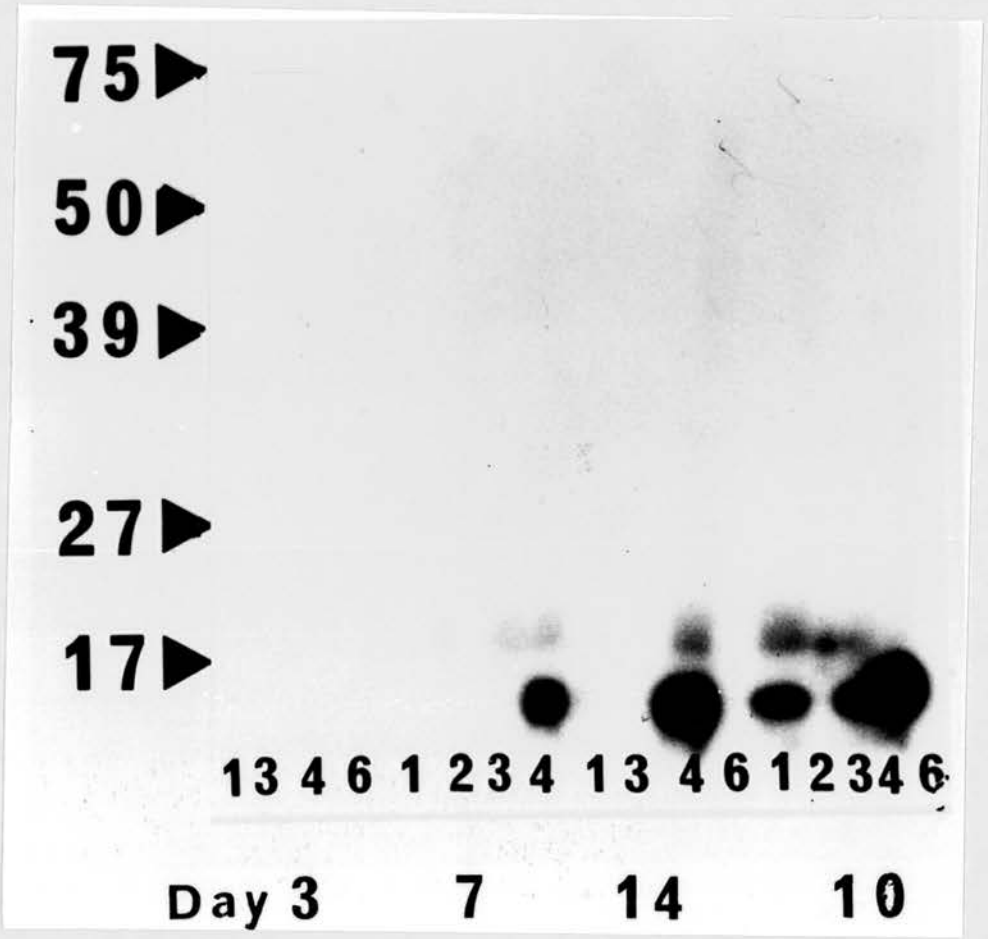
b) F344. Parasite-specific IgE was not present 3 days after secondary challenge but three rats (2, 3 and 4) responded on day 7 to an allergen in the 17,000 MW range (Fig. 4-5) and one animal (rat 4), responding to a 14,000 MW allergen, was the only one with detectable IgE on day 14. Parasite-specific IgE was produced by four of five rats tested after tertiary infection (day 21). Rats 1, 3 and 4 recognized 14,000 and 17,000 MW allergens and rat 2, the 17,000 MW allergen alone. Rat 6 did not respond at any stage and rat 5 was not tested.

Experiment II: Comparison of *N. brasiliensis*-specific IgE responses in LOU and Wistar rats using Western blotting and passive cutaneous anaphylaxis.

Protocol. For this experiment LOU rats were purchased from OLAC (Bicester, Oxon) because the MRI animal house was unable to supply sufficient numbers of this strain. Twenty six LOU and twenty six Wistar (this strain was chosen because it was to be used in a subsequent experiment described in Chapter 6) rats were infected subcutaneously with 3000 *N. brasiliensis* larvae and groups of 4-5

Figure 4-5

Western blot showing the secondary and tertiary IgE responses by F344 rats against adult *N. brasiliensis* worm antigens. Sample collection and timing of challenge were as described in Figure 4-4.



rats were killed by exsanguination under deep ether anaesthesia 0, 7, 10 and 14 days later. Worm burdens were counted on day 7 (Table 4-III). Sufficient time was left between primary and secondary infection to permit titres of parasite-specific IgE and concentrations of non-specific IgE to decrease. Thus, five animals from each strain were reinfected with 5,000 larvae 52 days after primary infection and were exsanguinated 14 days later. Sera collected in this manner were tested for parasite-specific IgE by Western blotting and PCA.

RESULTS

a) LOU. Parasite-specific serum IgE was demonstrable on day 7 of primary infection when all LOU rats responded, albeit poorly, to the 14,000 MW allergen (Fig. 4-6) and, on day 10, to allergens of 28,000, 32,000 and 50,000 MW, however the autoradiographs were very weak and difficult to reproduce photographically. Responses were similar on day 14 (Fig. 4-6) but parasite-specific IgE was more readily demonstrable after secondary infection. Rat 1 developed IgE antibodies against allergens of 14,000, 32,000, 50,000 and 69,000 MW (Fig. 4-7; Lane 6). Rat 2 (Lane 7, Fig. 4-7) responded similarly and to one additional allergen of 28,000 MW. Strong responses to 14,000 and 17,000 MW allergens by rats 3 and 4 and to the 50,000 MW allergen by rat 3 also occurred (Fig. 4-7; Lanes 8 and 9).

Table 4-III

Worm burdens in Wistar and LOU rats infected with *N. brasiliensis*

	Wistar*	LOU
	2148	2291
	2204	1863
	2098	2402
	1834	1767
	1727	1936
mean	2002	2052
+ SE	93	124

*Five animals of each strain were killed 7 days after infection with 3000 larvae and parasite burdens were counted.

b) Wistar. Wistar rats failed to mount serum IgE responses against low molecular weight allergens (14,000 and 17,000 MW) at any time after primary infection (Fig. 4-8) but did produce antibodies against 32,000-82,000 MW allergens. Parasite-specific IgE antibodies were present on day 7 when 3 rats responded to different allergens (including the 32,000 MW allergen). On day 10, two rats recognized the 32,000 MW allergen whilst two others failed to respond (Fig. 4-8). Three of four rats responded to varying degrees against the 50,000 MW allergen on day 14 (Fig. 4-8). In addition, each responding animal recognized two other allergens of relatively high molecular weight. Sera collected after reinfection contained IgE directed against 14,000 and 17,000 MW allergens and against a few of the high MW allergens described above (Fig. 4-8).

Figure 4-6

Western blot of AWH from *N. brasiliensis* showing parasite-specific IgE in serum from LOU rats undergoing a primary infection. The techniques used and the key to the figure are as described in Figure 4-1.

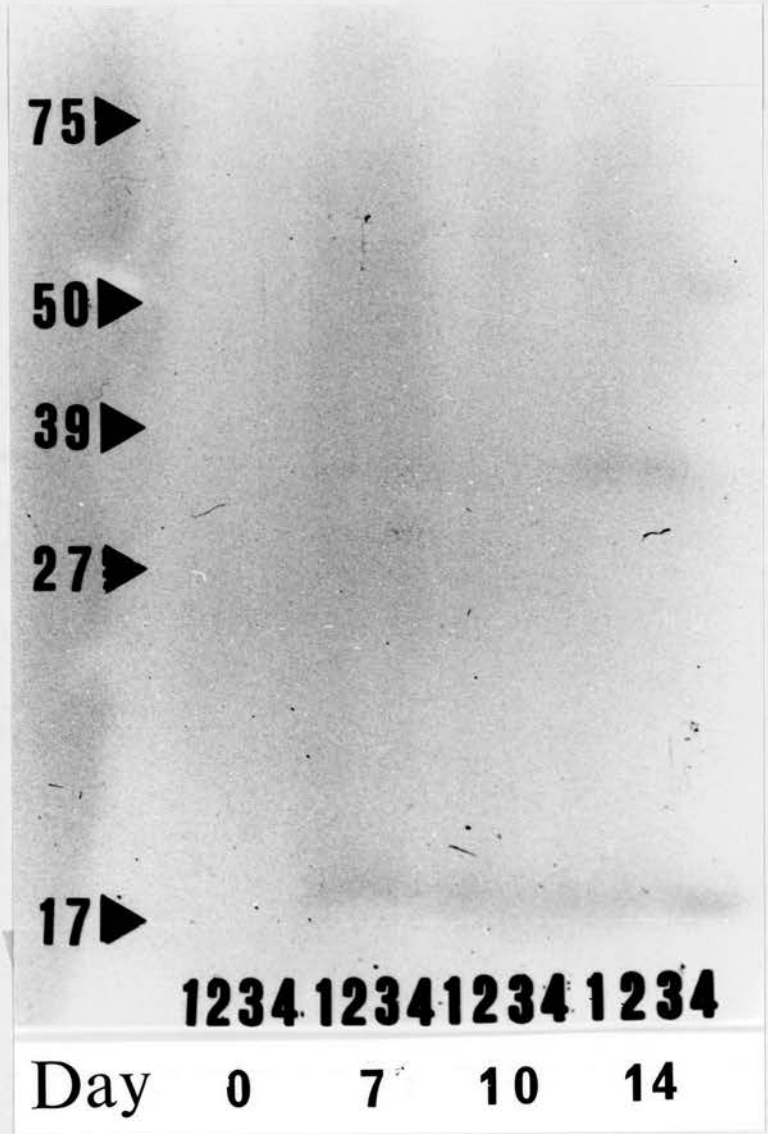
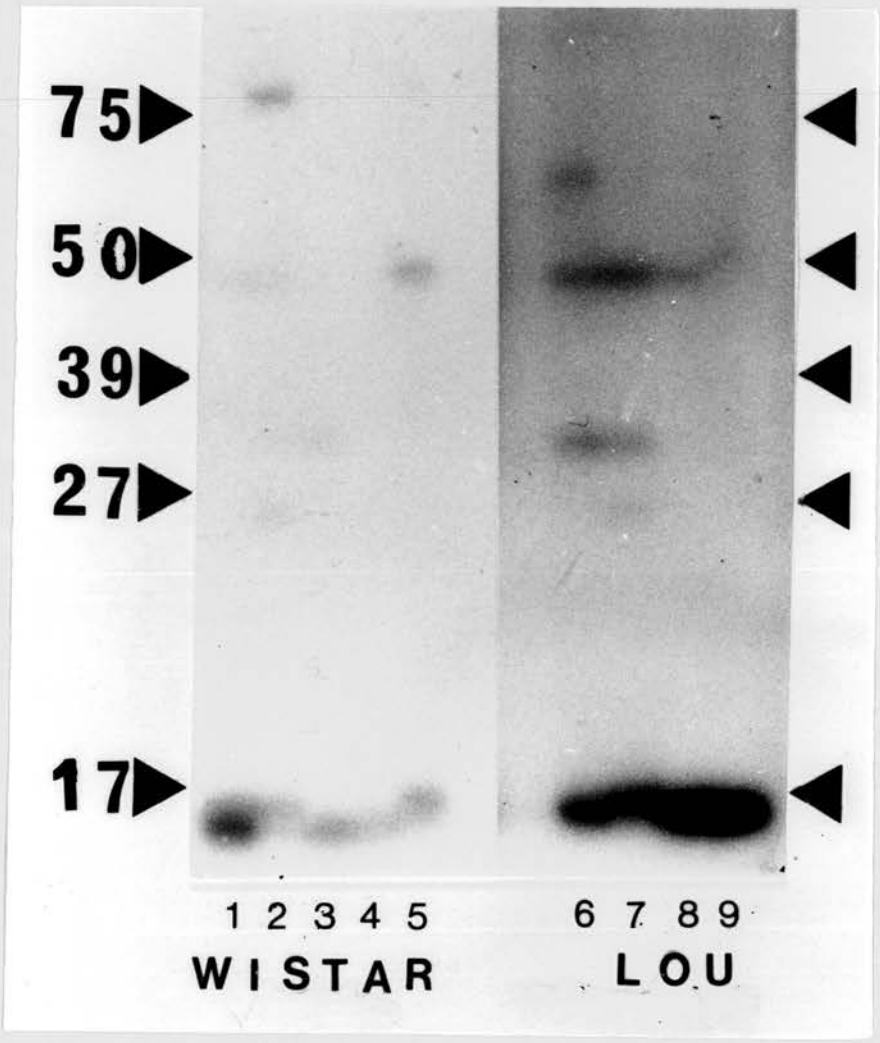


Figure 4-7

Western blot demonstrating parasite-specific IgE responses by Wistar rats during primary infection with *N. brasiliensis*. See Figure 4-1 for a description of the techniques used and for the key to the figure.

Figure 4-8

Western blots comparing the secondary IgE responses to adult *N. brasiliensis* worm antigens in Wistar and LOU rats. Both groups were challenged 52 days after primary infection and samples were taken 14 days later.



Analysis of LOU and Wistar Rat IgE responses by PCA. Samples of serum collected during the course of Experiment II were tested by passive cutaneous anaphylaxis (PCA). Responsiveness was first demonstrable in LOU serum 14 days after infection (Tables 4-III) but titres were low; PCA reactions were detected with undiluted serum from all five LOU rats tested. Higher PCA titres occurred after reinfection (1/300) although the results were complicated by an apparent prozoning effect where low serum dilutions produced no reaction whereas PCA occurred with serum dilutions of 1/200 and 1/300.

Serum collected from Wistar rats after primary infection lacked anaphylactic antibodies and only low PCA titres were demonstrable after reinfection (Table 4-IV). On day 14 after secondary infection, positive reactions were observed with undiluted serum from all rats but only three sera reacted at a dilution of 1/10 and none beyond this dilution (Table 4-IV).

TABLE 4.IV

Quantitation by PCA of IgE responses in LOU rats during primary and secondary infection with *N. brasiliensis*.

DAYS AFTER INFECTION	SERUM DILUTIONS	Diameter of reaction (mm) 1[a]	2	3	4	5	CONTROLS	
PRIMARY INFECTION								
Day 7	NEAT	-[b]	-	-	-	-	HIS[c]	17
	1/2	-	-	-	-	-	SALINE	-
	1/10	-	-	-	-	-	NRS[d]	-
Day 10	NEAT	-	-	-	-	-	HIS	14
	1/10	-	-	-	-	-	SALINE	-
	1/100	-	-	-	-	-	NRS	-
	1/200	-	-	-	-	-		
Day 14	NEAT	7	6	7	4	4	HIS	14
	1/10	-	-	-	-	-	SALINE	-
	1/100	-	-	-	-	-	NRS	-
	1/200	-	-	-	-	-		
SECONDARY INFECTION								
Day 14	NEAT	-	-	-	-	-	HIS	15
	1/10	-	-	-	-	-	SALINE	-
	1/100	-	-	-	-	-	NRS	-
	1/200	10	11	9	7	9		
	1/300	4	4	5	4	3		

Hooded Lister rats were sensitized by subcutaneous injection with serial dilutions of sera collected from LOU rats during experiment II. In addition, each animal was sensitized with hyperimmune and normal rat serum and with saline. Animals were challenged after 24 hours with 1000 w.e. in 1% EB.

Key to Table 4.~~3~~ III and 4 IV

[a] - rat number
[b] - denotes a negative reaction
[c] - hyperimmune serum (diluted 1/50)
[d] - Normal rat serum (diluted 1/50)

TABLE 4.V

Quantitation by PCA of IgE responses in Wistar rats during primary and secondary infection with *N. brasiliensis*

DAYS AFTER INFECTION	SERUM DILUTIONS	PRIMARY INFECTION Diameter of reaction (mm) 1[a]	2	3	4	5	CONTROLS	
DAY 7	NEAT	-[b]	-	-	-	-	HIS[c]	15
" "	1/2	-	-	-	-	-	SALINE	-
" "	1/10	-	-	-	-	-	NRS[d]	-
DAY 10	NEAT	-	-	-	-	-	HIS	14
" "	1/10	-	-	-	-	-	SALINE	-
" "	1/100	-	-	-	-	-	NRS	-
" "	1/200	-	-	-	-	-		
DAY 14	NEAT	-	-	-	-	-	HIS	17
" "	1/10	-	-	-	-	-	SALINE	-
" "	1/100	-	-	-	-	-	NRS	-
" "	1/200	-	-	-	-	-		
DAY 21	NEAT	-	-	-	-	-	HIS	13
" "	1/10	-	-	-	-	-	SALINE	-
" "	1/50	-	-	-	-	-	NRS	-
" "	1/100	-	-	-	-	-		
" "	1/200	-	-	-	-	-		
	SECONDARY INFECTION							
DAY 10	NEAT	-	-	-	-	-	HIS	8
" "	1/10	-	-	-	-	-	SALINE	-
" "	1/100	-	-	-	-	-	NRS	-
" "	1/200	-	-	-	-	-		
" "	1/300	-	-	-	-	-		
DAY 14	NEAT	5	12	12	11	4	HIS	14
" "	1/10	-	5	4	4	-	SALINE	-
" "	1/100	-	-	-	-	-	NRS	-
" "	1/200	-	-	-	-	-		

DISCUSSION

Six distinct allergens, from somatic extracts of adult *N. brasiliensis* worms, with molecular weights of 14,000, 17,000, 28,000, 32,000, 50,000 and 69,000 were demonstrable by Western blotting in experiments I and II. All six allergens were also identified in the preceding chapter (Chapter 3). There were some additional allergens against which Wistar rats had occasional and variable responses. The identification of several allergens in AWH by immunoblotting is consistent with previous studies on nematode allergens already discussed in the General Introduction. Two distinct allergens have been characterized from *N. brasiliensis* AWH (Wedrychowicz *et al.*, 1986) and there are many more as yet uncharacterized high molecular weight allergens in AWH (McWilliam *et al.*, 1987). Analysis of perienteric fluid has demonstrated a similar complexity for *A. suum* (McWilliam *et al.*, 1987). In contrast, *T. colubriformis* (Ford, 1971) and *T. spiralis* (Durham *et al.*, 1984; Santamarina *et al.*, 1988) apparently produce fewer allergens.

The specificity of the IgE produced by four rat strains was compared by Western blotting in the first series of experiments (Experiment I). Each group was given an identical challenge and, although worm burdens were similar in each group, the 4 strains responded differently. The allergens detected by different strains varied in molecular weight. For example, August and Hooded Lister rats recognized low molecular weight allergens (14,000 and 17,000 MW) and LOU rats detected both low (14,000 and 17,000 MW) and

higher (28,000, 32,000, 50,000 and 69,000 MW) molecular weight allergens. Wistar rats (Experiment II) initially responded to high and subsequently to low MW allergens (14,000 and 17,000 MW). During primary infection, the intensity and specificity of the IgE response varied between and within strains. For example LOU rats produce IgE specific for four allergens (14,000, 17,000, 50,000 and 69,000 MW) in almost all combinations. Interestingly, however, only the 14,000 and 17,000 MW allergens were recognized after reinfection, but again the intensity of the response was variable. Allergens of 50,000 and 69,000 MW, against which LOU rats responded during primary infection, were not detected after secondary infection in the first of 2 comparable experiments (compare Figures 4-1 and 4-4), whereas these allergens were recognised by LOU rats undergoing secondary challenge in the second experiment (compare Figures 4-4 and 4-8). The reasons for this discrepancy are not readily explained.

Variations in the intensity of IgE responses, measured by PCA, have been reported in earlier studies (Ogilvie and Jones, 1967). For example, sera collected from Sprague Dawley rats, 20 days after infection with 2500 *N. brasiliensis* L3 were tested by PCA. Some animals responded with PCA titres of 160-320 and other failed to respond or had very low titres (Ogilvie and Jones, 1967). This finding is not dissiimilar to many earlier studies of IgE responses to helminth infection (reviewed in Jarrett and Miller, 1982).

The faecal egg-counts of the four rat strains in experiment 1 were measured. There was an apparent correlation between low faecal egg counts and the detection of parasite-specific IgE in serum early during infection. F344 rats in particular, expressed the highest average faecal egg counts and were last to respond with anti-parasite IgE. In contrast, Hooded Lister rats responded first and had the lowest average faecal egg counts. This result is surprising in that Nawa and Miller (1979) noted that PVGc (Hooded Lister) rats were slower to expel *N. brasiliensis* than Wistar, DA and (DA x PVGc)F_1 strains.

The results in this chapter emphasize the already well-documented findings that the IgE response is variable (Revoltella and Ovary, 1969; Levine and Vaz, 1970; Pfeiffer, Konig and Bohn, 1983). As described earlier in the General Introduction, the IgE response is controlled by MHC Class II genes, by T cell-derived cytokines, and by the capacity to secrete IgE-binding factors which enhance or suppress IgE production. Cytokines and IgE-binding factors of non MHC-linked genes influence IgE responses (Wassom, *et al.*, 1983). Genetic influences on IgE synthesis are not well defined in rats although some strains have been described as high and low IgE responders based on simple comparisons. For example Hooded Lister (Meacock, Hilary and Marsden, 1976) and PVGc rats (Jarrett and Stewart, 1973a) were classified as high responders and Wistars as medium to low responders (Meacock *et al.*, 1976).

In the present study, variation in the IgE response, particularly within strains, has made it difficult to associate general response phenotypes with individual strains. Notwithstanding, some strain-associated response characteristics were apparent. For example, Hooded Lister rats responded earlier (on day 7 of infection) than the other strains tested in Experiment 1 and variation between rats was at a minimum with almost all Hooded Lister rats recognizing both 14,000 and 17,000 MW allergens. On the basis of these results and those of Meacock *et al.*, (1976) the Hooded Lister strain can be classified as an early IgE responder phenotype. Wistar and LOU rats also responded on day 7 (experiment 2) and, although the responses were weak and variable, these two strains can probably be classified as early responder phenotypes in this study although Wistars were previously classified as poor responders (Bennich and Johannson, 1971). However, PCA and Western blotting demonstrated quantitative and qualitative differences between the responses of LOU and Wistar rats. Parasite-specific IgE was not demonstrable in August rats until day 21 and this strain may be classified as a medium responder whereas F344 rats were low/late responders because anti-parasite IgE was demonstrable only after two infections with *N. brasiliensis* and the responses were also very variable. These results support previous comparisons (Jarrett and Stewart, 1973a; Meacock *et al.*, 1976) and extend them to include additional strains of rat. When secondary and tertiary IgE responses were evaluated, all strains tested responded at one time or another to the 14,000 and 17,000 MW allergens.

One feature of the IgE response, apparent in this study, was that some rats responded to low MW allergens on day 10 of primary infection but not on days 14 and 21. This pattern of response, observed in LOU rats (Experiment 1) and to a lesser extent in Wistar rats (Experiment 2) has not been observed before and is, again, difficult to explain. Hogarth-Scott (1973) proposed that circulating allergens block the PCA reaction and, if present, these might account for the present findings, but as yet *Nippostrongylus* allergens have not been detected in blood.

LOU rats were included in both experiments in this chapter and the infection regimes were varied between the two experiments. The IgE response recorded after secondary infection in experiment 2 was not dissimilar to that noted in chapter 3 (Fig. 3-1; Lanes 1-6) with high and low MW allergens identified. This is not surprising since similar infection regimes were implemented (primary infection with 3000 L_3 and secondary challenge with 5000 L_3). In contrast, when rats were infected first with 2000 L_3 and subsequently with 3000 and 5000 L_3, the secondary and tertiary IgE responses were restricted to the 14,000 and 17,000 MW allergens.

Parasite-specific IgE was demonstrable at an earlier stage of infection than has been documented in previous studies relying on PCA alone (Jarrett *et al.*, 1976; Befus *et al.*, 1982). This observation corroborates previous studies of the timing of the onset of tissue sensitivity in Hooded Lister rats, measured by active cutaneous anaphylaxis (Jarrett and Stewart, 1973a and

1973b). Similarly, sensitivity to parasite allergens in the trachea and jejunum of Lewis rat was detected on days 5 and 11 of infection respectively (Befus *et al.*, 1982). It was concluded that the early reactivity of the trachea correlated with the route of infection and with local synthesis of IgE antibody. Befus *et al.* (1982) proposed a model of sequestration whereby parasite-specific IgE, as soon as it is produced, is bound to high affinity IgE receptors, thus preventing its detection in serum.

Thoracic duct lymph (TDL) which drains the gut-associated lymphoid tissues (GALT) and other secondary lymphoid tissues contains antibody and cells derived from GALT. Allan and Mayrhofer, (1984) demonstrated parasite-specific IgE and IgE-bearing lymphocytes in TDL during infection with *N. brasiliensis*. Parasite-specific IgE was demonstrable in cultures of TDL lymphocytes collected on day 12, however PCA activity was not noted in the lymph until day 16. In contrast, it is quite clear from the present results that parasite-specific IgE is present in blood much earlier in infection than hitherto suspected from studies of PCA reactions.

The findings reported in this chapter therefore contradict previous studies using the PCA test which indicated that parasitic-specific IgE was not demonstrable in serum of some strains of rat prior to worm expulsion (Befus *et al.*, 1982; Alan and Mayrhofer, 1984; Jarrett *et al.*, 1976). However, a number of reports have noted the lack of sensitivity of the PCA test

(Butchko *et al.*, 1984; Jarrett, Mackenzie and Bennich, 1980) and Turner *et al* (1981) provided an example of how a sensitive immunoassay (RAST) could detect allergen-specific IgE which was undetectable by PCA. Rats were first immunized with ovalbumin in *Pertussis* adjuvant and subsequently infected with *N. brasiliensis*. Serum collected before and after infection was tested for ovalbumin-specific reagins. Anti-ovalbumin IgE antibody was clearly demonstrable before infection by both PCA and RAST, but could be detected only by RAST after infection. Similar observations were made by Jarrett *et al.*, (1980) who compared the respective sensitivities of radioimmunoassay (RIA) and PCA in the detection of ovalbumin-specific reagins; specific reagins were demonstrable by RIA but not PCA after *N. brasiliensis* infection.

Concentrations of non-specific IgE increase greatly during the early stage of infection with *N. brasiliensis* (Jarrett and Stewart, 1973; Jarrett *et al.*, 1976). The lack of sensitivity of the PCA test may be a consequence of high IgE concentrations and subsequent competition for mast cell IgE receptors (Godfrey, 1975). The zoning effect noted in Table 4-III may also be a consequence of competition for mast cell IgE receptors. For example, the PCA test was shown to be unsatisfactory when concentrations of total and non-specific IgE are raised during nematodiasis or atopy (Johansson, 1967; Berg and Johansson, 1969; Bennich and Johansson, 1971) and patients were refractile to passive sensitization (Godfrey and Gradidge 1976; Stanworth *et al.*, 1968). Van Tooreneberg, Aalberse and Reverinh-Brongers (1983) demonstrated *in vitro* the competitive effects of IgE binding.

In conclusion, therefore, comparisons of primary IgE responses to *N. brasiliensis* among the rat strains revealed differences in the specificities, magnitude and kinetics of the response of individual strains. These novel observations raise interesting questions about the genetic control of IgE responses and, in addition, *N. brasiliensis*-specific IgE was detected by immunoblotting at an earlier stage in primary infection than has been previously reported using the PCA test. The development of an IgE-blotting technique permits not only a sensitive and qualitative system for IgE detection but also eliminates the competitive effects of non-specific IgE which may reduce the efficacy of the PCA test.

CHAPTER 5

CATABOLISM OF RAT IgE AND IgG2a BY RAT MAST CELL PROTEASES I AND II

INTRODUCTION

Mast cells and basophils can be activated via a number of pathways, but most studies have concentrated on the IgE-dependent mechanism. IgE resides in a functional orientation on the surface of mast cells and basophils (Isersky, Rivera, Mims and Triche, 1979) and is bound by large numbers of monovalent, high affinity receptors (Sterk and Ishizaka, 1982; Lee, Sterk, Ishizaka, Bienenstock and Befus, 1985). Sensitized mast cells release inflammatory mediators when exposed to allergen or anti-IgE (Reviewed by Wasserman, 1983).

The temporal and functional relationships between antigenic or anti-IgE mediated stimulation, the relocation (patching) and endocytosis of surface IgE, and degranulation have been studied intensively using rat basophilic leukemia cells (RBL, a tumour analogue of basophils and mast cells) and rat myeloma IgE. When RBL and peritoneal mast cells were sensitized by incubation with monomeric I^{125}-IgE anti-Dinitrophenyl (DNP) (Feruichi, Rivera and Isersky, 1984) 50% of surface bound IgE was internalized rapidly (3 to 5 minutes) after cells were treated with DNP-carrier (Feruichi *et al.*, 1984). The rate and extent of internalization were almost unchanged in conditions of reduced receptor occupancy (Feruichi *et al.*, 1984). Although IgE dimers trigger mast cell endocytosis, larger oligomers are required for mast cell degranulation (Fewtrell and Metzger, 1980; Feruichi, *et al.*, 1984). It was also apparent from these studies that monomeric IgE and unoccupied

IgE-receptors were internalized with IgE oligomers (Isersky, Rivera, Segal and Triche, 1983; Feruichi, Rivera, Triche and Isersky, 1985). Some form of membrane receptor association may explain this observation (Feruichi *et al.*, 1985).

Little is known about the fate of internalized IgE but it is clear that, like IgG, IgE is internalized with its receptor (Feruichi *et al.*, 1984; Rivera, Mullins, Feruichi and Isersky, 1986) and remains associated with the receptor for long periods of time (Isersky *et al.*, 1983; Rivera *et al.*, 1986). Initially, internalized IgE was detected within 1 μm of the cell surface membrane (Isersky *et al.*, 1983) a region rich in small unit-membrane-bound vesicles and thought to be involved in intracellular transport (Ishizaka and Ishizaka, 1974). Although IgE and IgG aggregates are internalized at a similar rate, preliminary biochemical and ultrastructural studies indicate that the intracellular fate and processing of IgG and IgE aggregates may differ (Rivera *et al.*, 1986).

When concentrations of serum IgE are high, IgE is demonstrable within MMC but not CTMC (Mayrhofer *et al.*, 1984; Lindsay, Blaies and Williams, 1984; Hickling and Greenwood, 1989). The present study was conducted to determine whether the biochemical differences between RMCP I and II could, in part, explain the differential distribution of IgE in mast cell subsets. The

capacities of RMCP I and RMCP II to cleave rat IgE were examined. For comparative purposes, another rat immunoglobulin class, IgG2a, was chosen because it is reaginic in the rat and might be exposed *in vivo* to the mast cell proteases.

Experimental Protocol

The immunoglobulin substrates for RMCP I and II, rat IgE and IgG2a, were diluted and dialysed against PBS pH 7.5 with 0.5M NaCl and 0.01% azide. In order to standardize the reactions and to compare them with other studies an enzyme/substrate ratio of 1:10 was chosen for RMCP II. Values were calculated in nanokatals/milligram (nk/mg) of protein as described previously (Knox, Gibson and Huntley, 1986). Therefore, for RMCP II the ratio of 1:10 was achieved when 1nK enzymic activity was added to 3.4μg immunoglobulin. Because RMCPI had a much lower activity, a much higher concentration of protease to substrate was required (see below).

Digestion of IGE by RMCP I and II: 74 nanokatals of RMCPI (132 μg) and RMCPII (25 μg) were each added to 252 μg IgE to achieve equivalent ratios of enzyme activity to substrate. After incubation at 37^{o}C for 0.5, 5, 10, 30 and 60 minutes or for 24 hours, aliquots of 25 μg immunoglobulin were removed. Each was diluted 1:1 in reducing sample buffer before analysis by SDS-PAGE.

Additional aliquots (15 μg) were removed for PCA analysis at 0.5, 30 and 60 minutes and 24 hours after digestion and diluted 1:1 in NRS to inhibit further chymotryptic activity (Pirie-Shepherd, Miller and Ryle, 1991).

Digestion of IgG2a by RMCP I and II: 79 nanokatals of RMCPI and II were each added to 270μg of IgG2a to achieve equivalent ratios of enzyme activity to substrate. After incubation at 37^{o}C for 0.5, 5, 10, 15, 30 and 60 minutes and 6 and 24 hours, aliquots were removed and diluted 1:1 in reducing sample buffer and then analysed by SDS-PAGE.

Control IgE and IgG preparations were treated in the same manner, but not exposed to enzyme. Samples were removed at 0.5 and 60 minutes and at 6 (for IgG) and 24 hours, and treated as above. Results were assessed by SDS-PAGE, Western blotting and PCA (for IgE). Western blots were probed with ^{125}I-labelled mouse monoclonal anti-rat IgE (Sera-lab, Sussex, U.K.)or with a rabbit anti-rat IgG (H and L chain) horseradish peroxidase conjugate (Dakopatts, Glostrup, Denmark).

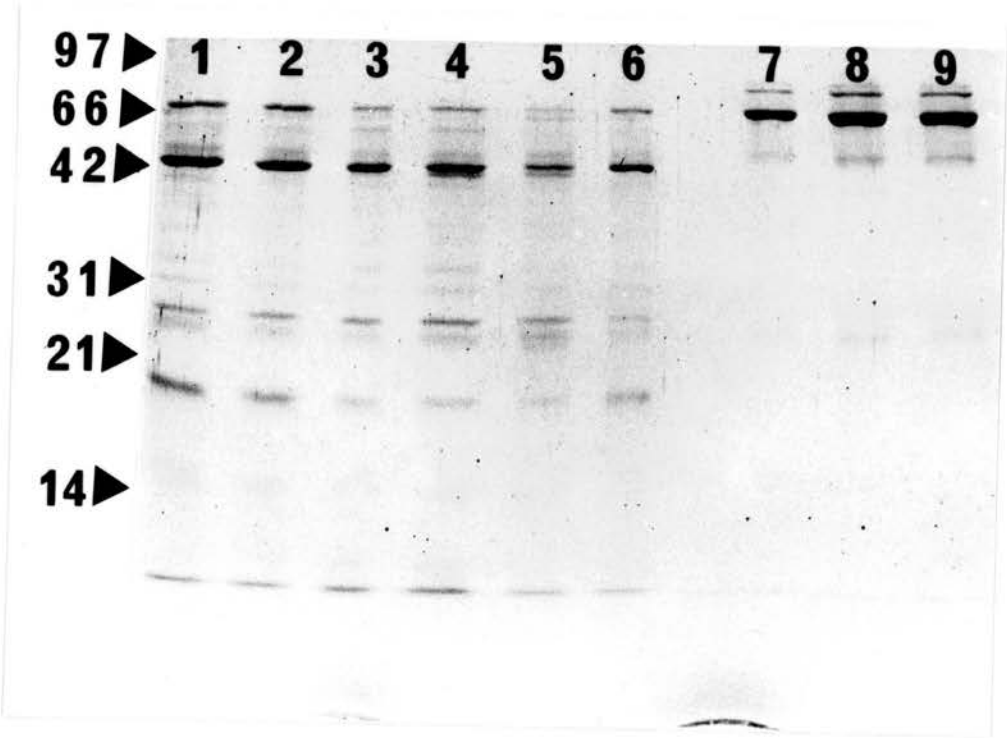
RESULTS

Digestion of IgE by RMCP I

Partial degradation of the Epsilon chain (MW 66,000) was observed within 30 seconds of addition of RMCP I, and seventeen peptides with molecular weights ranging from 64,000, to 18,000

Figure 5-1 a and b

The catabolism of polyclonal rat IgE by RMCP I is demonstrated:- (a) by SDS-PAGE and (b) by Western blotting. Serum from Hooded Lister rats twice immunized by infection with *N. brasiliensis* was the source of IgE. The latter was purified by affinity chromatography and mixed with RMCP I (1nK/enzyme per 3.4 μg IgE) at 37^{o}C. Samples (25 μg) were taken 30 seconds, 5, 15, 30 and 60 minutes, and 24 hours later (lanes 1-6 respectively) and subjected to SDS-PAGE under reducing conditions. Control IgE preparations incubated in the absence of RMCP I were sampled after 30 seconds, 60 minutes and 24 hours and subjected to SDS-PAGE (lanes 7-9). The blot (b) was probed with radio-iodinated monoclonal anti-rat IgE. The molecular weight markers are described in Material and Methods.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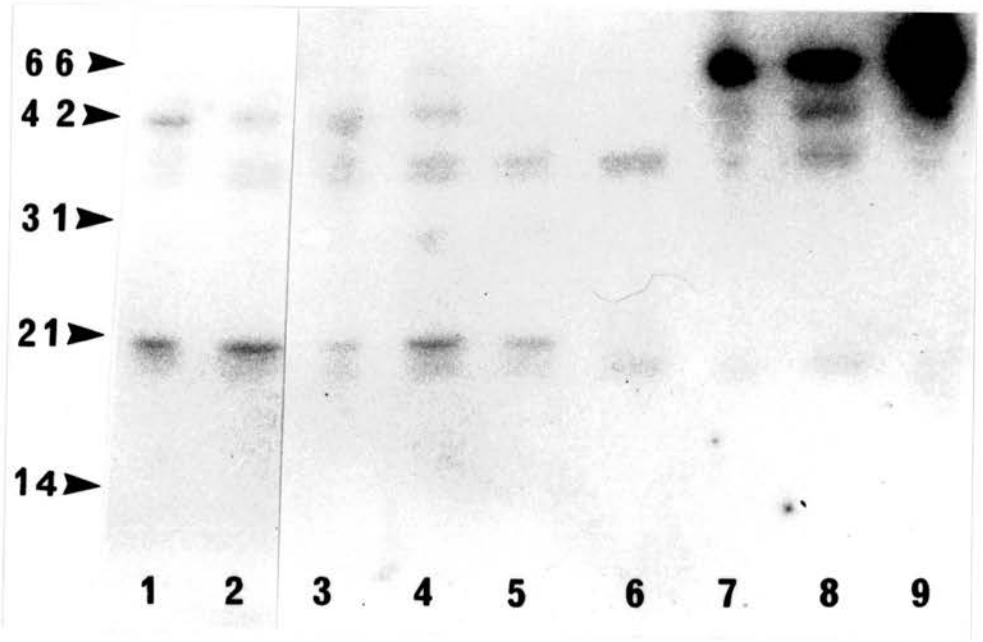
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were demonstrable by SDS-PAGE (Fig. 5-1a). No further significant degradation was obvious in Coomassie-stained gels after 24 hours. Control samples in which the IgE heavy chain was the predominant polypeptide, incubated in the absence of RMCPI, were unchanged over 24 hours (Fig. 5-1a).

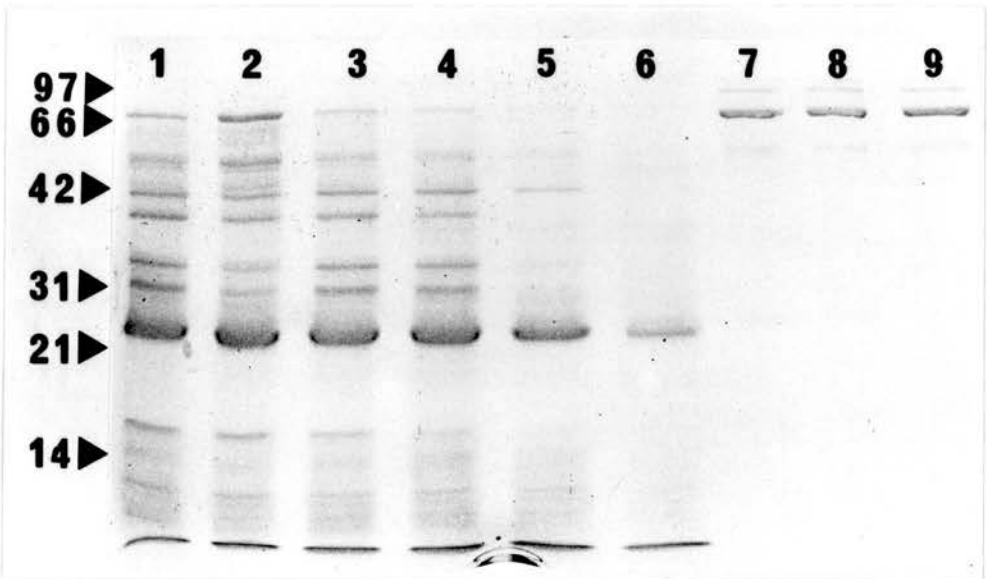
By Western blotting, the Epsilon chain was intensely labelled in control preparations (lanes 7-9) but was barely detectable in the samples treated with RMCPI (Fig. 5-1b, lanes 1-6). Four peptides of MW 43,000, 36,000, 21,000 and 19,000 were demonstrable with monoclonal anti-rat IgE 30 seconds after addition of RMCPI. The 43,000 and 21,000 MW peptides disappeared after 60 minutes and 24 hours respectively, but antigens of 36,000 and 19,000 MW were still present after 24 hours (Fig. 5-1b). In addition to the 66,000 MW heavy chain in control samples, two peptides with molecular weights of 42,000 and 36,000 were identified by anti-rat IgE and all were still demonstrable after 24 hours (Fig. 5-1b, lanes 7-9).

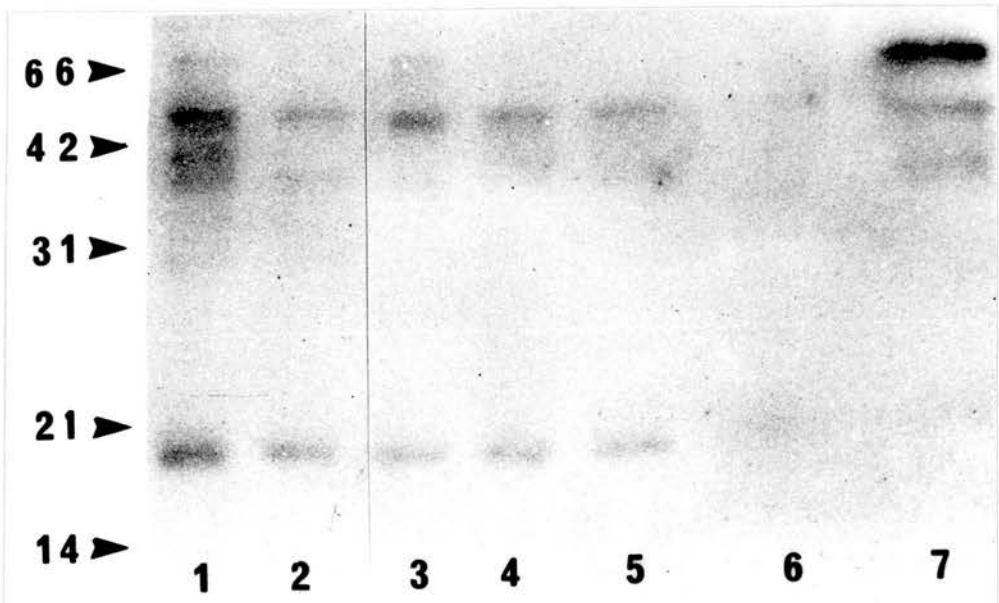
Digestion of IgE by RMCP II

Addition of RMCP II to affinity purified polyclonal rat IgE resulted in the rapid partial cleavage of the Epsilon heavy chain into 14 peptides with MWs ranging from 65,000 to less than 10,000 (Fig. 5-2a). Comparison of lanes 1 to 6 indicated that catabolism continued throughout the period of incubation with a gradual loss

Figure 5-2 a and b

Degradation of polyclonal IgE by RMCP II demonstrated (a) by SDS-PAGE and (b) by Western blotting. Sampling times and labelling as for Figures 5-1a and b except that lane 7 in the Western blot (b) contains a control sample of untreated IgE.





of higher molecular weight peptides and a total degradation of the Epsilon chain by 24 hours. A peptide of approximately 23,000 MW was particularly abundant and was still present, although slightly diminished in staining intensity, after 24 hours incubation.

By Western blotting, the 66,000 MW epsilon heavy chain was virtually undetectable after 30 seconds and 2 additional antigens with molecular weights of approximately 43,000 and 20,000 were evident at 6 hours (Fig. 5-2b, lanes 1-5) but were barely detectable at 24 hours (lane 6).

Functional Assessment of IgE Degradation Products by PCA

The *in vivo* functional activity of affinity-purified IgE was assessed by PCA (Table 5-1). Treatment of IgE with RMCP I or II caused an immediate reduction in PCA reactivity. Only a slight reduction was observed in control preparations.

Digestion of IgG2a by RMCP I

After 30 seconds treatment with RMCP I peptides of 42,000, 40,000 and 35,000 MW were derived from the IgG_{2a} H chain (Fig. 5-3a). These were present at low concentrations initially but were more prominent after 60 minutes when there was a simultaneous decrease in staining intensity of the heavy chain. A number of peptides of less than 20,000 MW were also first detected at 60

Table 5-I

Functional activity of IgE after catabolism by RMCP I or II measured by PCA.

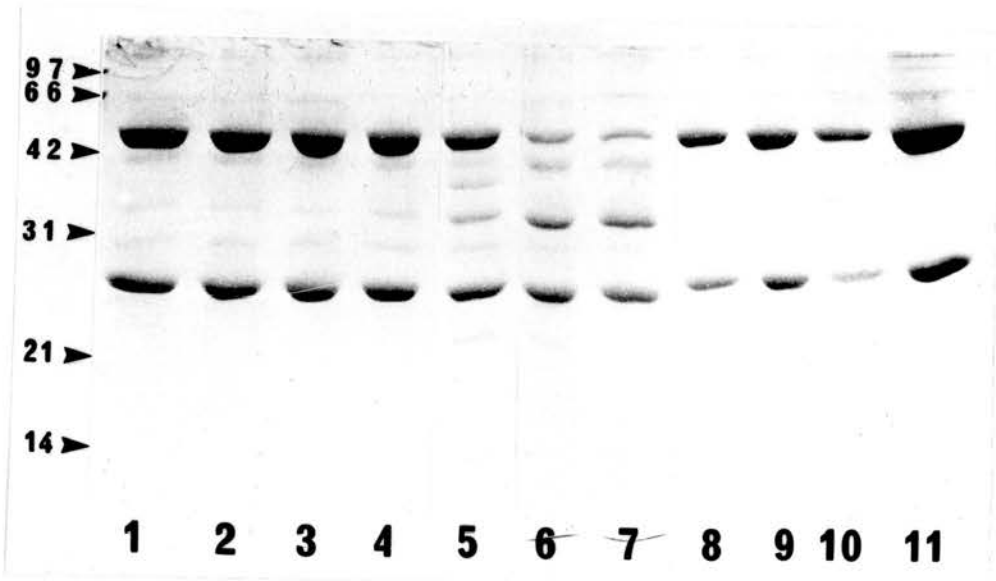
Time[a]	Diameter in mm			
	Experiment One			
	Control IgE	RMCPI/IgE	RMCPII/IgE	Control[b]
0 min	15 17[c]	10 11	9 6	Saline 0
30 min	15 15	2 2	4 2	NRS[d] 0
60 min	12 11	0 0	0 0	HIS[e] 15
24 hours	12 12	0 0	0 0	
Time	Experiment Two			
0 min	18 16[c]	14 13	12 12	Saline 0
30 min	18 16	10 8	10 4	NRS[d] 0
60 min	17 16	0 0	0 0	HIS[e] 12
24 hours	16 14	0 0	0 0	

Four male Hooded Lister rats were used in each experiment. Animals were sensitized by subcutaneous injection with IgE collected from a single time-point in the experiment. In addition, each rat was sensitized with hyperimmune serum, normal rat serum and saline. Animals were challenged 24 hours later by intravenous injection of 1000 worm equivalents in 1% Evans Blue and killed by exsanguination under ether, after a further 2 hours.

a period of incubation with protease.
b control samples.
c duplicate test on the same animal.
d normal rat serum (mean).
e hyperimmune rat serum (mean).

Figure 5-3a and b

Digestion of IgG2a by RMCP I analysed by SDS-PAGE (a) and by Western blotting (b). Affinity purified monoclonal rat IgG_{2a} was catabolized by RMCP I and aliquots (5 μg) were harvested at 30 seconds, 5, 15, 30 and 60 minutes, and 6 and 24 hours (Lanes 2 to 7) together with control IgG_{2a} preparations which were recovered at 30 seconds, 1, 6 and 24 hours (lanes 8 to 11) and were compared by SDS-PAGE. Gels were either stained with Coomassie blue (a) or blotted and probed with polyclonal rabbit anti-rat IgG (H+L) peroxidase conjugate (1/200) (b) Molecular weight markers are as described in Materials and Methods.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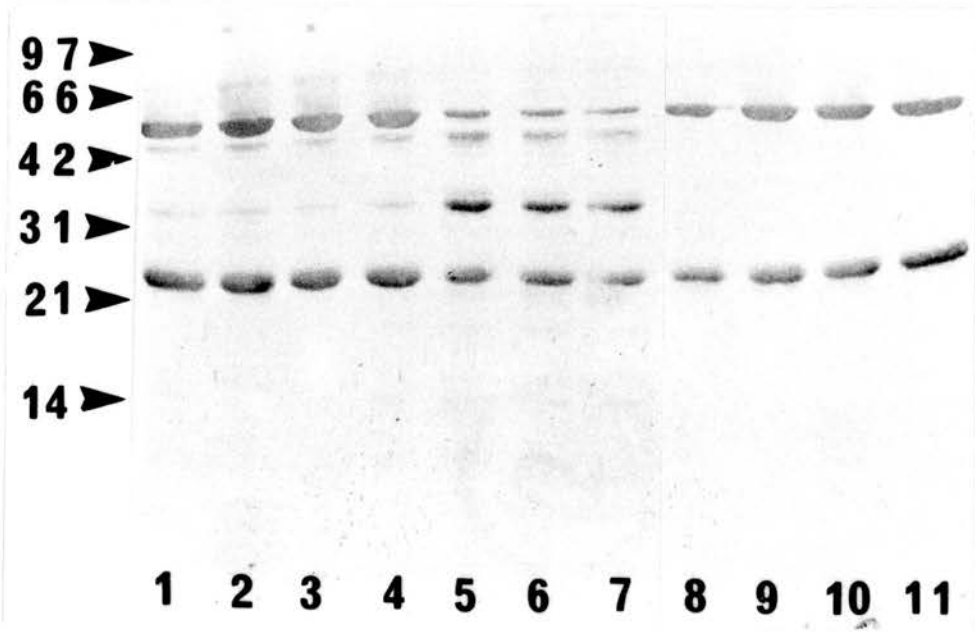
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minutes. Both the H chain and its degradation products were still present after 24 hours. Control preparations incubated in the absence of enzyme remained intact. The IgG_{2a} light chain was not apparently catabolized by RMCP I (Fig. 5-3a).

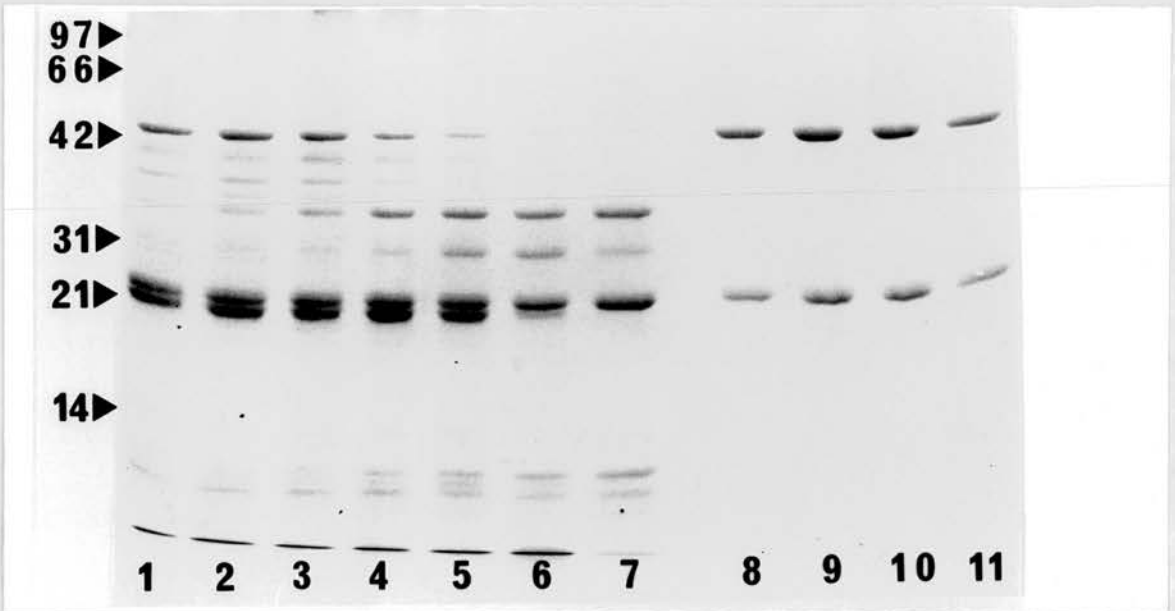
Although H chain digest fragments were demonstrable by SDS-PAGE after only 0.5 minutes, they were first identified on Western blot after a 5 minute digestion (Fig. 5-3 b) when 49,000 and 40,000 MW peptides as well as intact H + L chains were detectable. The 49,000 and 40,000 MW peptides were no longer prominent after 1 hour. After 24 hours incubation, the intensity of labelling of both heavy chain and digest products was markedly reduced.

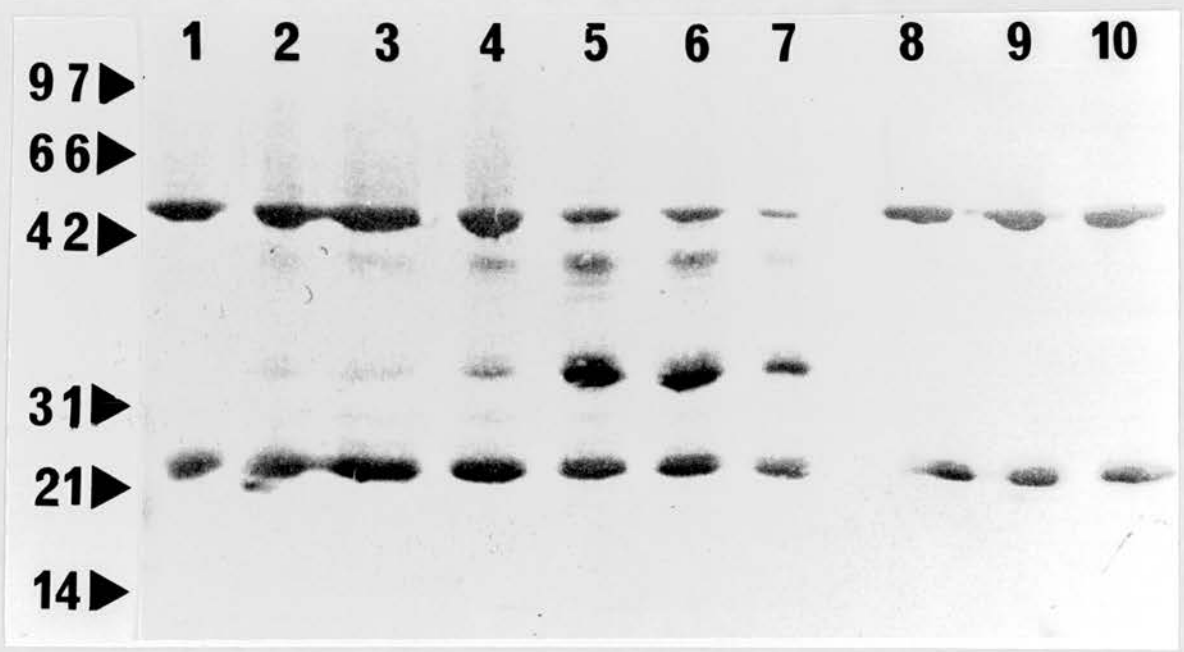
Digestion of IgG2a by RMCP II

Within 30 seconds of the initiation of the reaction, RMCP II had cleaved the IgG2a heavy chain to produce at least seven peptides ranging in MW from 42,000 to 9,000 and with a particularly strong band at 21,000 MW. Degradation continued and, after 24 hours, the heavy chain and all but four peptides (35,000, 29,000, 10,000 and 9,000 MW) were completely degraded. However the light chain (23,000 MW) was apparently resistant to digestion. Control IgG preparations incubated in the absence of RMCP II were unaltered after 24 hours (Fig. 5-4 a).

Figure 5-4 a and b

Catabolism of rat IgG2a by RMCP II analysed by SDS-PAGE (a) and Western blotting (b). For details of protocol and key to figures see Fig. 5-3 a and b.





Some of the peptides described in Coomassie blue-stained gels were also detected by Western blotting. Digestion products were demonstrable within 30 seconds, together with a concomitant decrease in staining intensity of the heavy chain (Fig. 5-4b) which was further reduced at 60 minutes and was undetectable thereafter. Digestion products increased gradually in concentration until 60 minutes and, subsequently, bands at 33,000, 29,000 were resistant to further degradation. The staining intensity of the heavy chain declined slowly until 60 minutes and was undetecable thereafter (Fig. 5-4b).

DISCUSSION

The results in this chapter show that both RMCPI and II were able to cleave rat IgE but that the rates of proteolysis and the cleavage products were markedly different. RMCPII acted more rapidly, causing an almost immediate degradation of Epsilon chain within 30 seconds. Fourteen peptides were detected by SDS-PAGE, one of which, with a molecular weight of 23,000 was abundant early in the reaction and was still demonstrable though in much reduced quantities, after 24 hours. It is likely that the 21,000 MW product on SDS-PAGE and the 19,000 MW antigen detected in the blot are the same. Small differences in the mobility of the markers may account for the observed discrepancy in molecular weight. Rapid proteolysis was also noted with RMCPI, producing seventeen peptides

within 30 seconds of commencing digestion, some of which were recognized by anti-IgE on Western blot. Degradation was less rapid at subsequent time-points and the Epsilon chain was still present after 24 hours digestion.

The relevance of *in vitro* studies of this type to the *in vivo* fate of IgE within mast cells is open to question. In mast cells RMCP I is tightly bound by granule heparin. This association is known to impose stoichemical restrictions on the activity of RMCP I within the granules such that only lower MW polypeptides (< 17,000 MW) are degraded (Le Trong *et al.*, 1989). If, after internalization, IgE complexes are transported to the secretory granule compartment, RMCP I is likely to have limited access to such a large molecule. There is, however, evidence to suggest that RMCP I remains associated with granule heparin even after secretion (reviewed in Stevens, 1989). By contrast RMCP II is freely secreted by MMC (Miller *et al.*, 1990) and is, therefore, less strongly associated with granule proteoglycans. Thus, RMCP II could digest immunoglobulin either internalized within the granules or, once secreted, on the surface of mast cells, assuming that extracellular plasma and tissue-derived inhibitors did not immediately prevent proteolysis. Further studies are therefore needed to determine the role of both rat mast cell proteases in IgE degradation.

The present results also confirm previous work, showing that the substrate specificities of RMCP I and II although similar in some respects are quite distinct (Woodbury *et al.*, 1989). When tested

against pulmonary epithelial cells grown *in vitro* on 'cytodex', RMCP II, at picomolar concentrations, caused permeability changes whereas RMCP I was effective only at much higher concentrations (Woodbury, *et al.*, 1989). Importantly, RMCP I caused cell death and damage whereas the cultured cells recovered rapidly and retained integrity within 24 hours of treatment with RMCP II (Woodbury *et al.*, 1989). *In vivo*, it is likely that RMCP II contributes to the changes in mucosal permeability observed during mucosal mast cell activation (Miller *et al.*, 1986). These results suggest that the *in vivo* functions of both proteases are likely to be distinct which may also explain the different tissue distribution of each protease (Miller *et al.*, 1990).

In addition to the distinct degradation products produced by digestion of IgE by RMCP I and II, further differences in specificity were apparent from studies of the catabolism of IgG_{2a}. RMCPII cleaved the gamma heavy chain quickly, resulting in several peptide products and with the complete loss of heavy chain at the end of the time-course. In contrast, RMCPI acted more slowly and produced fewer degradation products and the gamma chain remained detectable at the end of the experiment. This *in vitro* analysis of a native substrate therefore further emphasized the distinction between RMCP I and II.

The exact fate of IgE in mast cells has yet to be defined. In macrophages, internalized immunoglobulin is transported together with the Fc receptor to the lysosomal compartment where it is degraded rapidly. Small non-TCA-precipitable products are released

from the cell less than 20 minutes after internalization commences (Mellman and Plutner, 1984). In contrast, IgE and IgG complexes internalized by RBL (Rat Basophilic Leukaemia cell) and isolated peritoneal mast cells, are apparently degraded and released more slowly (Rivera *et al.*, 1986). RBL cells accumulated and processed the cross-linked immunoglobulin before releasing it to the extracellular environment. IgG was released at a rate of less than 3%/hour, slower than that for IgE (greater than 10%/hour). It was also noted that 95% of the released IgG but only 50% of the released IgE were precipitated by trichloroacetic acid (TCA) (Rivera *et al.*, 1986).

Previous studies have established that mast cells adsorb exogenous materials such as colloidal gold and poly-L-lysine (Padawer, 1970; 1986), which were subsequently found within the secretory granules. It is conceivable that internalized IgE is transported in a similar manner to the secretory granules. Observations made during helminth infection (Mayrhofer *et al.*, 1976; Lindsay, *et al.*, 1984) detected intracellular IgE in MMC but not CTMC. The precise location of the internalized IgE in relation to the granules is not clear but since both RMCP I and II can cleave IgE it is unlikely that the observed difference in IgE staining reflects the specific activities of the respective enzymes. Futher advances in this work will require ultrastructural analysis of the distribution of internalized IgE, a goal which will be difficult to achieve given the difficulties in locating IgE immunohistochemically in fixed cells (Mayrhofer *et al.*, 1976).

This is not the first time the effects of mast cell-derived proteases on IgE were assessed. Coutts, Nehring, Jariwala, Weiryb and Khandwala (1981) described proteolysis of IgE by putative proteases released during purification of rat mast cells by density gradient centrifugation. The proteases involved were not, however, identified.

The proteolytic effects of trypsin, chymotrypsin, pepsin and papain on IgE have been assessed previously (Brown and Lee, 1976; Kolmannskog, 1987 and Kolmannskog, Marhaug and Haneberg, 1985), where faeces from patients with atopy or with clinical gut disorders were examined and, in many cases, an IgE fragment of 40,000 MW was identified in faecal extracts (Kolmannskog, 1987). This fragment expressed the E_{D1} determinant found on the the Fc portion of IgE. The purified fragment was called Fc'' and appears to be resistant to further degradation by either the faecal enzymes or chymotrypsin. An Fc-like fragment was generated from purified human IgE after chymotrypsin treatment. This fragment, like Fc'', had a molecular weight of 40,000 MW, expressed the E_{D1} determinant and was resistant to further degradation (Kolmannskog, 1987).

There is some evidence for IgE heterogeneity in this study. A rapid reduction in Epsilon chain staining intensity was noted within 30 seconds of RMCPI treatment. However the Epsilon chain was still intact at the end of the experiment. This may indicate heterogeneity within the Epsilon chain and a resistance to degradation by one or more IgE isoforms.

Using a number of IgE myeloma proteins, Lehrer, McCants, Farris and Bazin (1981) demonstrated heterogeneity in the Fc portion of human IgE. Individual myeloma proteins expressed distinct capacities to sensitize mast cells and it was apparent from competitive inhibition studies that some myeloma proteins could competitively inhibit sensitization with some but not all myeloma proteins. Rheiner and Zahner (1986) noted that the levels of parasite-specific IgE measured by ELISA did not correspond to the level demonstrable by PCA. They concluded that this discrepancy may reflect diversity in the IgE isotype and differences in affinity for the IgE receptor. IgE heterogeneity has also been found in other species including the rat (Godfrey, Gradidge, Hawksley and Elliot, 1978).

RMCP I and II both cleaved the immunoglobulin substrates, IgE and IgG. It is unlikely that the specificity of these enzymes explains the detection of intracellular IgE in MMC but not CTMC. This study also confirms previous observations that RMCP I and II express distinct specificites. Although both enzymes cleaved substrates, the digest products and rates of proteolysis were distinct.

CHAPTER 6

ANALYSIS OF IGE-BEARING CELLS IN BM, PBL AND PERITONEAL LAVAGE DURING INFECTION WITH *NIPPOSTRONGYLUS BRASILIENSIS*

INTRODUCTION

A variety of cell types of haemopoietic origin express cell surface IgE receptors ($Fc_{\varepsilon}R$). However, historically, the function of IgE has primarily been associated with mast cells and basophils (Stanworth *et al.*, 1967; Ishizaka and Ishizaka, 1970; Sullivan, Grimsley and Metzger, 1971). Much of the current understanding of the biology of IgE has been obtained using monoclonal myeloma proteins (reviewed by Bazin and Pauwells, 1982). Characterization of ileocaecal immunocytomas in LOU rats has facilitated functional and biochemical studies (Bazin, Querinjean, Beckers, Hermans and Dessy, 1974).

Mast cells, basophils and rat basophilic leukaemia cells (RBL) a mast cell analogue, express as many as 90,000 IgE receptors (Fc_{ε} RI) per cell (Metzger and Bach, 1978; Ishizaka, Sota and Ishizaka, 1973). $Fc_{\varepsilon}RI$ is a univalent and high affinity receptor (Alcaraz, Kinet, Liu and Metzger, 1987). This explains why low concentrations of IgE can sensitize mast cells and RBL for prolonged periods of time (Isersky *et al.*, 1979). However IgE is not bound irreversibly. Using RBL, it was apparent from *in vitro* studies that 30-40% of cytophilic IgE dissociated from $Fc_{\varepsilon}RI$ (Isersky *et al*, 1979).

Eosinophils, platelets, monocytes, macrophage, T cells, B cells and NK cells bind IgE (Capron, Kusniertz, Prin, Spiegelberg, Khalife, Tonnel and Capron, 1985; Joseph, Capron, Ameiseu, Capron, Vorng, Pancre, Kusniertz and Auriault, 1986; Pancre,

Cesbron, Auriault, Joseph, Chandenier and Capron, 1988; Pestel, Joseph, Dessaint and Capron, 1988; Gonzalez-Molina and Speigelberg, 1977; Yodoi and Ishizaka, 1979; 1980; Melewicz and Spiegelberg, 1977) via a distinct low affinity receptor ($Fc_{\varepsilon}RII$) which is antigenically and structurally distinct from $Fc_{\varepsilon}RI$ (Melewicz, Plummer and Spiegelberg, 1982). *In vitro* $Fc_{\varepsilon}RII$ cells attach to and damage helminth parasites (Capron *et al.*, 1985; Pestel *et al.*, 1988) but their role *in vivo* has not been explored.

Leucocytosis, a feature of nematode infection, is indicative of a strong bone marrow response and is reflected, to some extent, in the peritoneal cavity (Rothwell and Love, 1975). Because of their potential role in helminth immunity cells from peripheral blood, bone marrow, and the peritoneal cavity were examined by flow cytometry to determine the presence of IgE-bearing cells during and after infection with *N. brasiliensis*. It was anticipated that this very sensitive technique would throw further light on the overall kinetics of IgE-bearing cells as well as providing more limited information on the size and complexity of these cells prior to their recruitment to the tissues.

Experimental Procedure

Twenty five Wistar rats were infected with 3000 *N. brasiliensis* L3. Five rats were killed by exsanguination under deep ether anaesthesia on each of the following days:- 0, 5, 10, 15 and 25. Blood, collected into heparin-containing tubes, was the source of PBL, and peritoneal cells were collected in RPMI-1640 medium by

peritoneal lavage as described (Chapter 2). Bone marrow cells were collected from the femur (Chapter 2). Peripheral blood leukocytes, bone marrow, and peritoneal lavage cells were washed three times in RPMI-1640 and tested for viability using trypan blue dye exclusion. Cells were resuspended in a small volume RPMI-1640 medium with 5% FCS and counted. They were maintained at $4^{o}C$ throughout. Aliquots of 2×10^{6} cells from each compartment were treated with MARE-1 FITC or, for control purposes, with an unrelated monoclonal antibody VPM-FITC (as described in the Materials and Methods Chapter 2), and analysed by FACScan and UV microscopy (Chapter 2). In addition, cytocentrifuge preparations from all three compartments were stained with Leishman's stain and analysed by light microscopy. Erythroid cells encompassed erythroblasts, normo blasts and reticulocytes; granulocytes included neutrophils, eosinophils and basophils and their precursors, small lymphoid cells were classed as lymphocytes and large lymphocytes/monocytes as large monuclear cells. Statistical analyses in this chapter were performed by analysis of variance and the student's t-test using the minitab computer programme.

Kinetics of different cell populations in bone marrow, peripheral blood and peritoneal cavity during infection with *N. brasiliensis*

The proportion (and range) of the different cell populations in each of the 3 compartments are shown in Tables 6-I, 6-II and 6-III. When compared with values for day 0, the most obvious changes in bone marrow occurred on days 15 and 25 of infection

where the proportions of small lymphoid cells, cells of the erythroid series, and 'other' cells declined, whereas granulocytes and their precursors increased, as did the proportion of large mononuclear cells (Table 6-I).

In peripheral blood, the fluctuations in cell populations were relatively small (Table 6-II) and were characterized by a decline in the proportion of lymphocytes on day 10 ($P < 0.001$) with a commensurate increase in the neutrophil population ($P < 0.001$) and in large mononuclear cells ($P < 0.02$). A slight eosinophilia occurred during infection and was maximal on day 25 (Table 6-II).

The changes in peritoneal lavage cells were most obvious on day 10 when there was a reduction in the proportion of lymphocytes ($P < 0.20$) which was associated with recruitment of large monuclear cells/macrophages ($P < 0.02$). On day 25 the mast cell and eosinophil populations were increased (Table 6-III).

RESULTS

Quantification and partial Characterization by flow cytometry of IgE-bearing cells in bone marrow, peripheral blood, and peritoneal cavity during infection with *N. brasiliensis*

Cells were analysed on dot-plots (FSC v SSC) (Figs. 6.1, 6.2 and 6.3) and analytical gates encompassing cells differing in size (FSC) and complexity (SSC) were based on criteria defined by

TABLE 6-I

Distribution of cell types in bone marrow analysed after Leishman staining of cytocentrifuge preparations

	0[a]	5	10	15	25
Small lymphoid[b] cells	92[c]	100	108	64	84
	(68-100)[d]	(84-108)	(52-116)	(40-96)	(72-100)
	7.5[e]	7.8	8.4	4.9	6.5
	23[f]	25	27	16	21
Erythroid cells	116	124	92	80	80
	(92-112)	(104-148)	(52-124)	(60-104)	(48-112)
	9.3	9.6	7.1	6.2	6.2
	29	31	23	20	20
Mast cells	0	8	4	4	4
	(0-4)	(0-20)	(0-8)	(0-8)	(0-8)
	0	0.6	0.3	0.3	0.3
	0	2	1	1	1
Large mononuclear cells	68	64	80	104	96
	(56-80)	(56-76)	(68-84)	(72-108)	(60-100)
	5.3	4.9	6.2	8.1	7.4
	17	16	20	26	24
Granulocyte	116	88	96	144	136
	(92-140)	(76-100)	(60-140)	(128-184)	(136-140)
	9.3	6.8	7.4	11.2	10.5
	29	22	24	36	34
Other	8	16	20	4	0
	(0-8)	(4-32)	(16-44)	(0-4)	(0-4)
	0.6	1.2	1.5	0.31	0
	2	4	5	1	0

a Time in days after infection with *N. brasiliensis*
b Cell type by Leishman stain
c Mean cell count from 5 rats
d Range
e Mean cells per femur ($x10^6$ cells)
f Mean percentage cells

TABLE 6-II

Distribution of cell types in PBL following cytocentrifuge preparation and Leishman stain.

	0[a]	5	10	15	25
Lymphocytes[b]	268[c]	264	140	276	252
	(240-316[d])	(248-250	(72-180)	(228-300)	(212-244)
	6.7[e]	23.1	8.4	13.1	17.6
	67[f]	66	35	69	63
Eosinophils	8	8	12	20	24
	(0-16)	(4-12)	(8-20)	(12-20)	(12-28)
	0.2	0.7	0.7	0.9	1.6
	2	2	3	5	6
Neutrophils	36	56	112	24	32
	(32-56)	(52-60)	(76-124)	(8-40)	(20-44)
	0.9	4.9	6.7	5.1	2.2
	9	14	28	6	8
Large mononculear cells	84	72	136	80	88
	(40-116)	(60-88)	(112-188)	(44-120)	(60-108)
	2.1	6.3	8.1	3.8	6.2
	21	18	34	20	22
Basophils	4	0	0	0	4
	(0-6)	0	0	0	(0-7)
	0.1	0	0	0	1.1
	1	0	0	0	1

a Time in days after infection with *N. brasiliensis*
b Cell type by Leishman stain
c Mean cell count from 5 rats
d Range
e Mean cells per rat PBL ($x10^6$ cells)
f Mean percentage cells

TABLE 6.III

Distribution of cell types in peritoneal lavage analysed following cytocentrifuge preparation and Leishman stain.

	0[a]	5	10	15	25
Lymphocyte[b]	140[c]	136	44	56	88
	(128-172[d])	(68-208)	(40-60)	(37-76)	(80-104)
	5.6[e]	7.8	2.8	5.2	6.4
	35[f]	34	11	14	23
Eosinophil	36	36	24	72	72
	(16-44)	(24-36)	(12-44)	(52-104)	(56-80)
	1.4	2.1	1.5	6.7	5.0
	9	9	6	18	18
Neutrophil	16	16	12	8	8
	(0-28)	(0-28)	(0-20)	(0-12)	(0-12)
	0.6	0.9	0.7	0.7	0.6
Basophil	4	4	0	4	8
	(0-8)	(0-8)	0	(0-8)	(0-12)
	0.2	0.2	0	0.4	0.6
	1	1	0	1	2
Macrophage/monocyte	188	188	292	212	188
	(172-196)	(80-264)	(284-300)	(184-268)	(156-188)
	7.7	11.0	18.3	20.7	13.4
	48	48	73	56	48
Mast Cells	12	8	28	36	28
	(8-16)	(12-20)	(20-36)	(20-44)	(20-36)
	0.5	0.5	1.7	3.3	1.9
	3	2	7	9	7

a Time in days after infection with *N. brasiliensis*
b Cell type by Leishman stain
c Mean cell count from 5 rats
d Range
e Mean cells per rat peritoneal lavage ($x10^6$ cells)
f Mean percentage cells

Figure 6.1

FACScan analysis of rat bone marrow: FSC verses right angle SSC light scatter dot plot. Dead cells and a proportion of red blood cells were gated out at the time of sample analysis (< 50 FSC scale live gate). Analytical gates were applied when samples were recalled from computer memory. The FSC vs SSC plot and the location of analytical gates were consistent from one bone marrow sample to another.

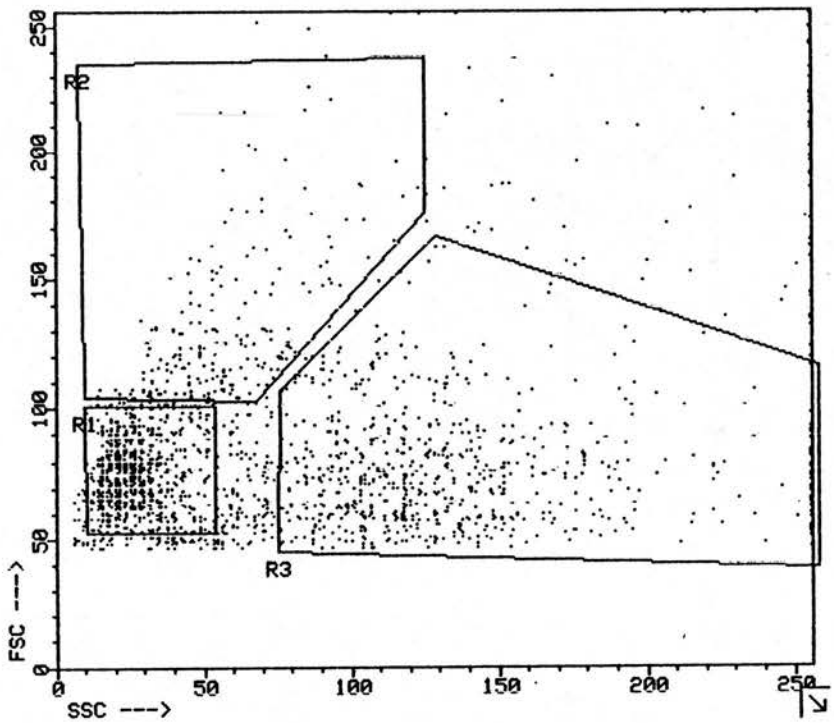
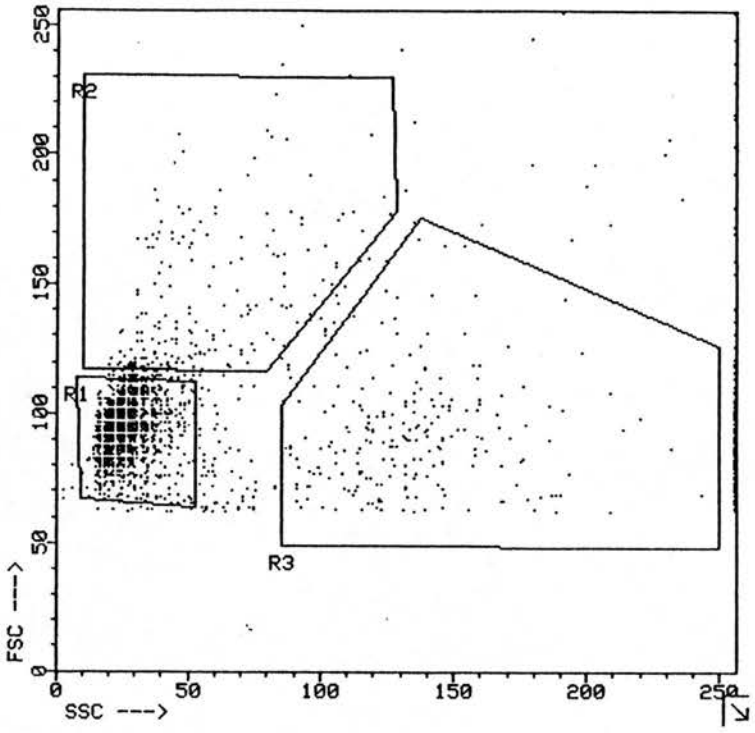

R2
R1
R3
FSC --->
SSC --->
0
50
100
150
200
250

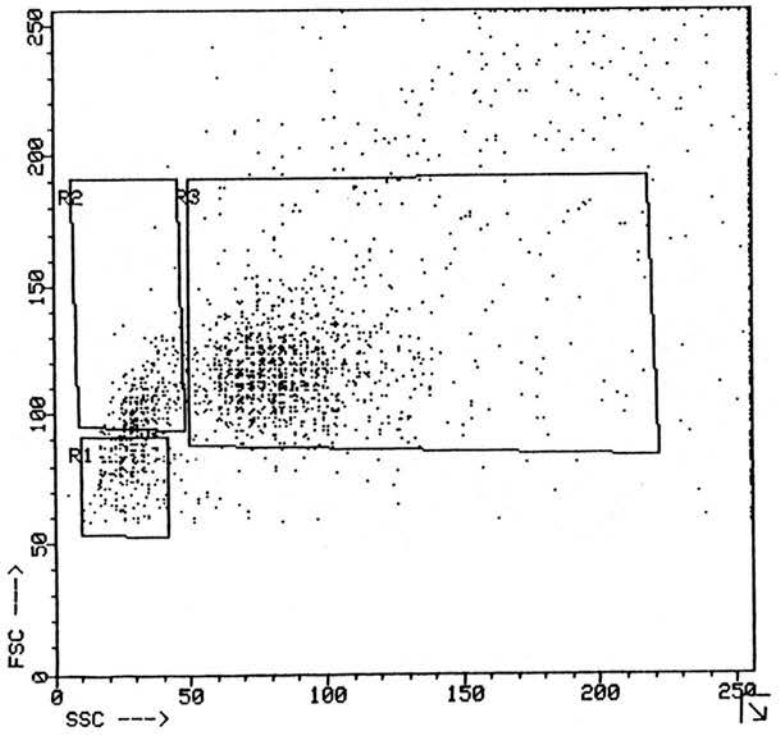
Figure 6.2

FACScan analysis of rat peripheral blood leukocytes: FSC verses right angle SSC light scatter dot plot. The procedures followed are outlined in the legend to Fig. 6.1.

Figure 6.3

FACScan analysis of rat peritoneal lavage cells: FSC verses right angle SSC light scatter dot plot. See Fig. 6.1 for the procedures used.


R2
R1
R3
FSC --->
SSC --->
0
50
100
150
200
250


R2
R3
R1
FSC --->
SSC --->
0
50
100
150
200
250

Loken, Shah Dattilio and Civin (1987); Haig, Brown and Thompson (1990) and Haig, Thomson and Dawson, 1991. Slight variations in forward and side scatter for whole BM cell populations were sometimes noted and minor adjustments to the gates were sufficient to accommodate these changes.

Three gates were set:- gate 1, which comprised cells of low forward and low side scatter. It is clear from previous studies (Haig *et al.*, (1991) that small lymphocytes (PBL, BM and peritoneum) and small erythroid series cells (BM) predominate in this gate. Gate 2, defined an area of low SSC and high FSC populated by medium to large lymphocytes, monocytes, macrophages (PBL, BM and peritoneum) and haemopoietic progenitor cells (BM) (Haig *et al.*, (1991). Gate 3 contains cells with high SSC and includes mast cells and other complex granulocytic cells such as neutrophils and eosinophils (BM and PBL) (Haig *et al.*, 1990).

Day 0 and 5 of infection. Despite the heterogeneity of BM cells from normal, uninfected rats (day 0) 41% were encompassed within gate 1. Gates 2 and 3 accounted for 9% and 33% of cells in this analysis (Fig. 6.1). Similarly, in peripheral blood, gate 1 contained the largest proportion of cells (66%) with gates 2 and 3 accounting for 5% and 21% of cells, respectively (Fig. 6.2).

For peritoneal lavage, in contrast with both the BM and PBL, gate 1 contained the smallest population of cells (19%). The majority were located within gate 2 and 3 (43% and 20% respectively) (Fig. 6.3). Generally the FACScan results on the

proportions of cells in any region compare well with the frequencies of defined cell types determined on Leishman's stained cytosmears (see Tables 6-1, 6-2 and 6-3). The predominant cell types in peritoneal lavage were mononuclear cells and macrophages and this is reflected by the high proportion of cells in gate 2.

IgE-bearing cells in bone marrow, blood and peritoneal cavity following infection with *N. brasiliensis*

Day 0 and 5

BM, PBL and peritoneal lavage cells harvested on day 0 were treated with MARE-FITC to detect surface IgE or, for control purposes, VPM-FITC or saline as described (Chapter 2). Cells were subsequently analysed by flow cytometry (FACScan) and by fluorescence microscopy (UV-M). There were no significant differences between saline and VPM-FITC control treatments at any stage or in any compartment (results not shown). The proportions of IgE-bearing cells in BM, peritoneal lavage, and peripheral blood were very low or undetectable on day 0 (Figs 6.4 to 6.7). Similar results were demonstrable by fluorescence microscopy. There were no significant changes in the populations of IgE-bearing cells in BM or PBL on day 5 (Fig. 6.8 to 6.10), although there was a significant reduction in the proportion of IgE-bearing cells in PBL ($P < 0.05$) (Fig. 6.4) and an increase though not significant in peritoneal lavage (Fig. 6.4)

Figure 6.4

Quantitation of IgE-bearing cells during the course of infection with *N. brasiliensis*. Values shown are mean ± SE of total ungated, FITC-labelled cells identified by Flow Cytometry. The parameters used for the detection are described in figure 6.5

FIG. 6.4 Total IgE-bearing cells in BM, PBL and Peritoneum

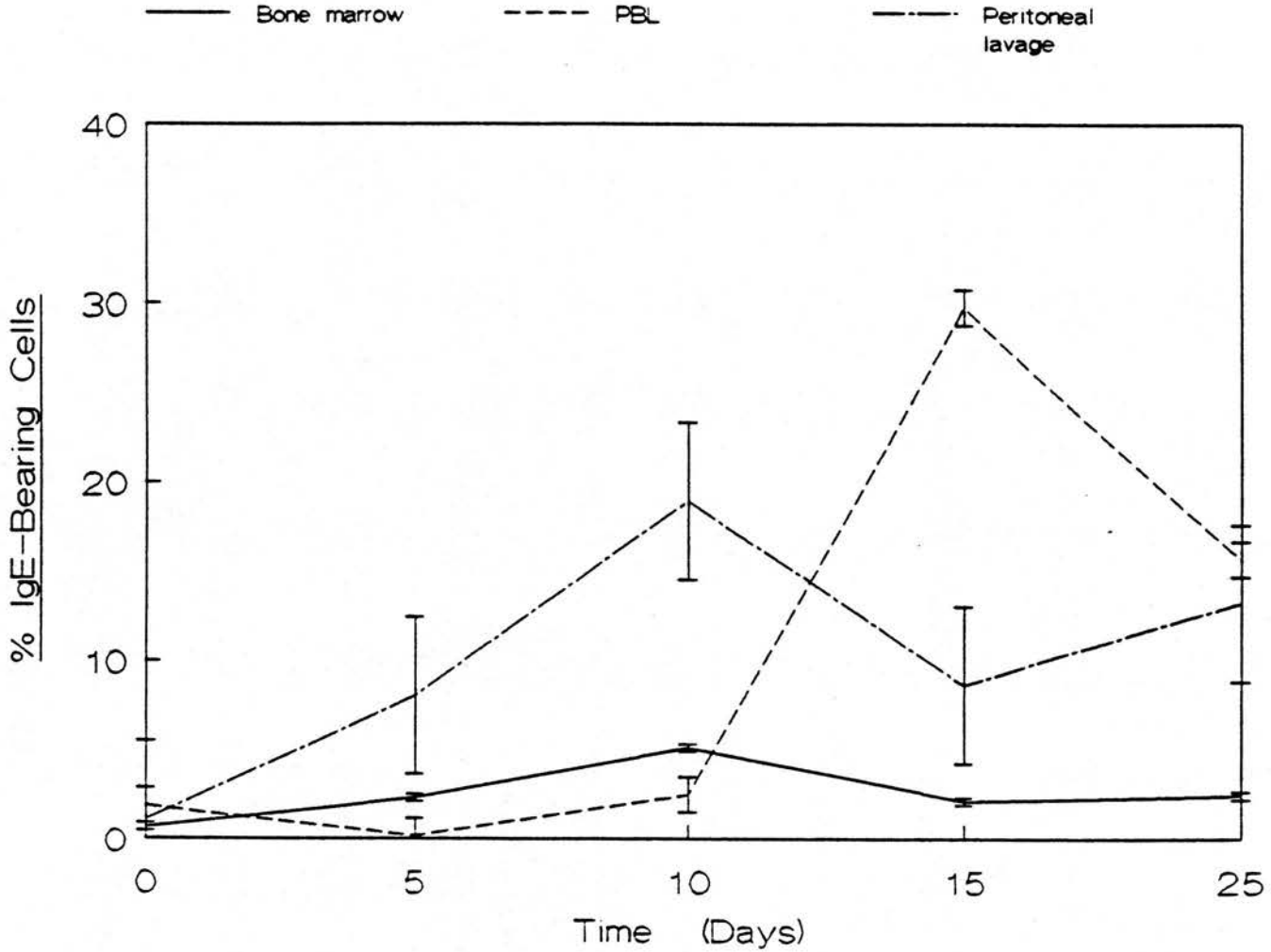
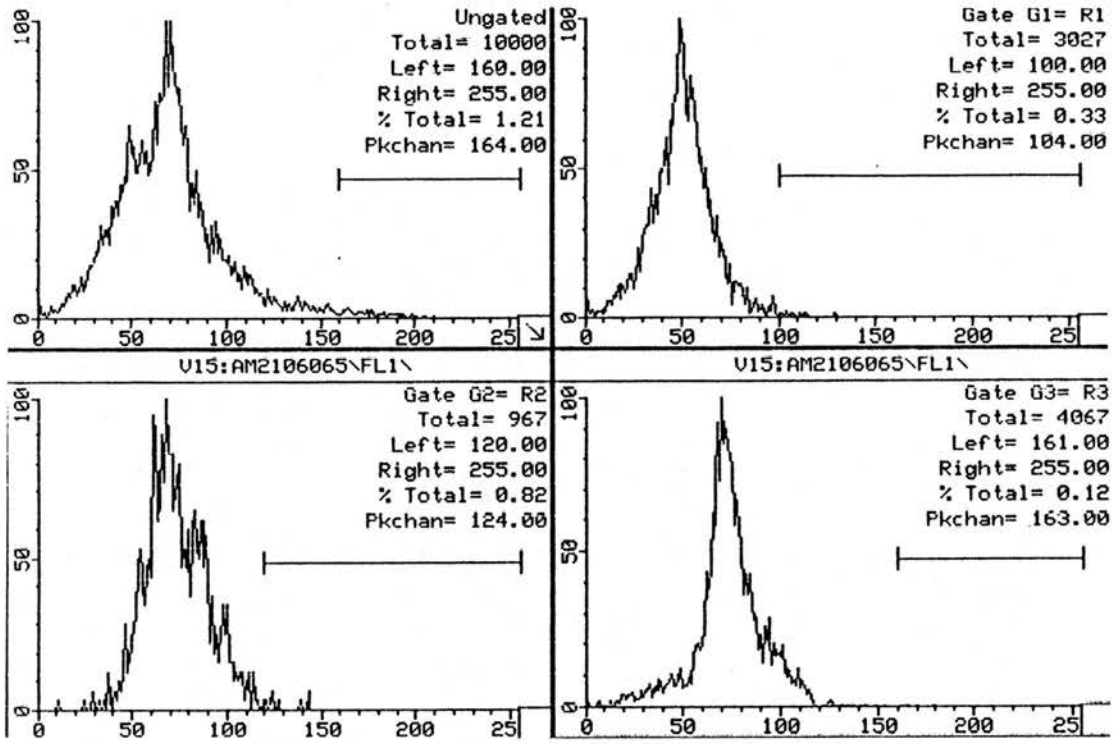


Figure 6.5

Isolated bone marrow cells from a normal uninfected rats were were labelled with MARE-FITC (top) or VPM-FITC (bottom) (5 μg/ml) and 10,000 cells were analysed for fluorescence by FACScan (described in Chapter 2). Results are shown as log fluorescence (horizontal axis) against cell numbers (vertical axis). BM cells were analysed as a total population (ungated) (see Fig. 6.4) and as subpopulations based on size and complexity (as already discussed in this Chapter) Gate 1, Gate 2 and Gate 3. Bars are aligned on each histogram to indicate areas containing positive cells.


Ungated
Total= 10000
Left= 160.00
Right= 255.00
% Total= 1.21
Pkchan= 164.00
Gate G1= R1
Total= 3027
Left= 100.00
Right= 255.00
% Total= 0.33
Pkchan= 104.00
V15:AM2106065\FL1\
V15:AM2106065\FL1\
Gate G2= R2
Total= 967
Left= 120.00
Right= 255.00
% Total= 0.82
Pkchan= 124.00
Gate G3= R3
Total= 4067
Left= 161.00
Right= 255.00
% Total= 0.12
Pkchan= 163.00

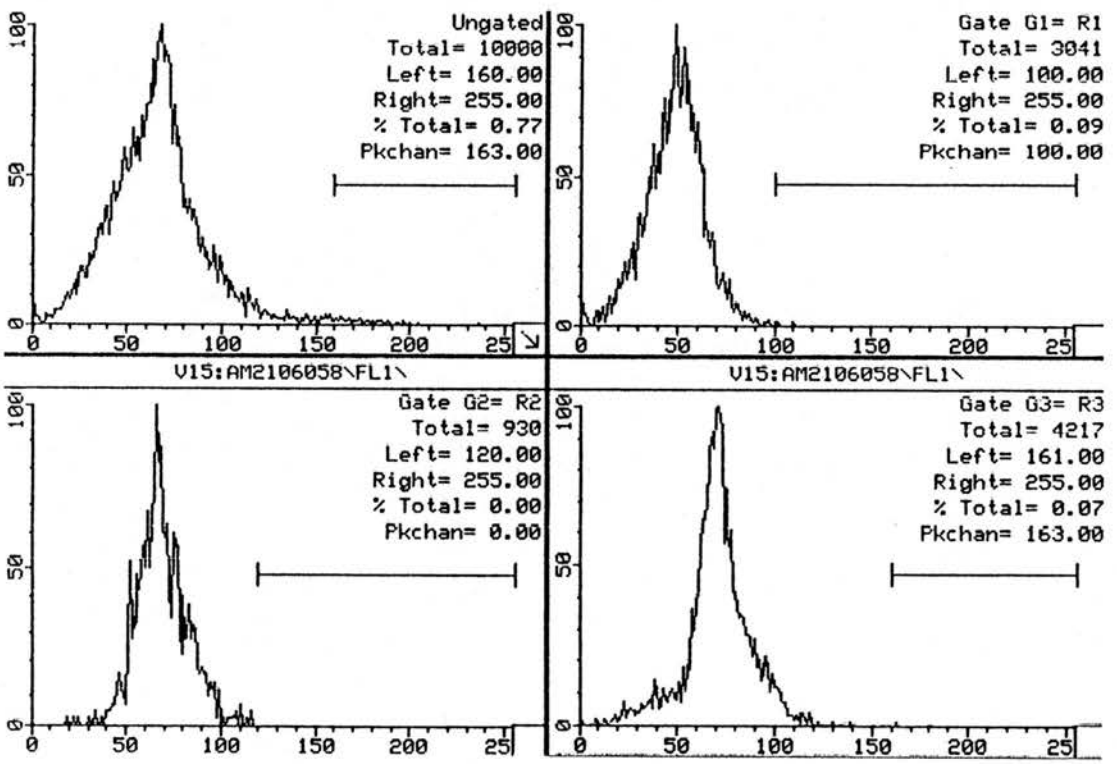

Ungated
Total= 10000
Left= 160.00
Right= 255.00
% Total= 0.77
Pkchan= 163.00
Gate G1= R1
Total= 3041
Left= 100.00
Right= 255.00
% Total= 0.09
Pkchan= 100.00
V15:AM2106058\FL1\
V15:AM2106058\FL1\
Gate G2= R2
Total= 930
Left= 120.00
Right= 255.00
% Total= 0.00
Pkchan= 0.00
Gate G3= R3
Total= 4217
Left= 161.00
Right= 255.00
% Total= 0.07
Pkchan= 163.00

Figure 6.6 and 6.7

Peripheral blood (6.6) and peritoneal cells (6.7) from normal animals were labelled as described in Figure 6.5 and parameters shown are as described in Figure 6.5

Figure 6.6

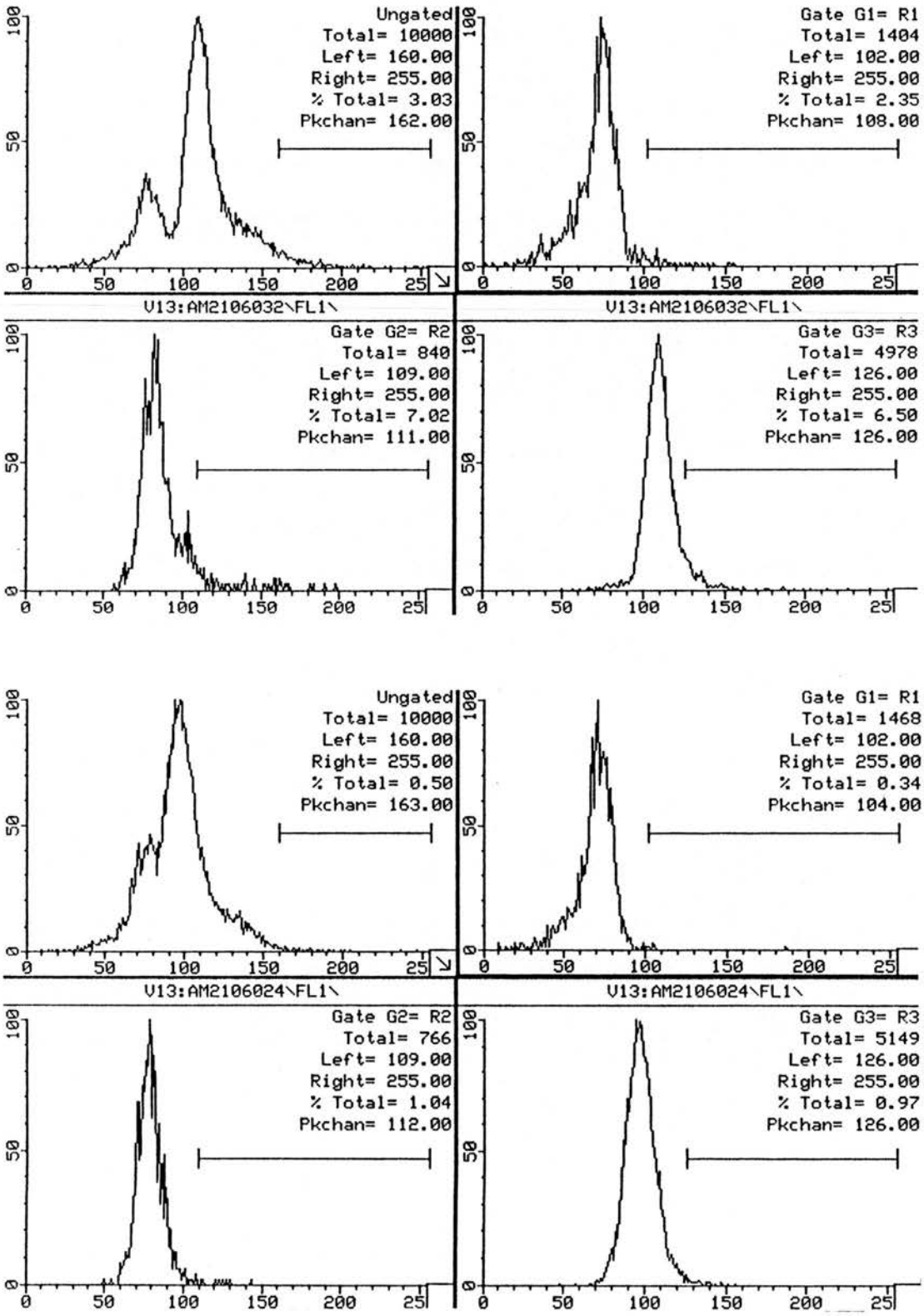
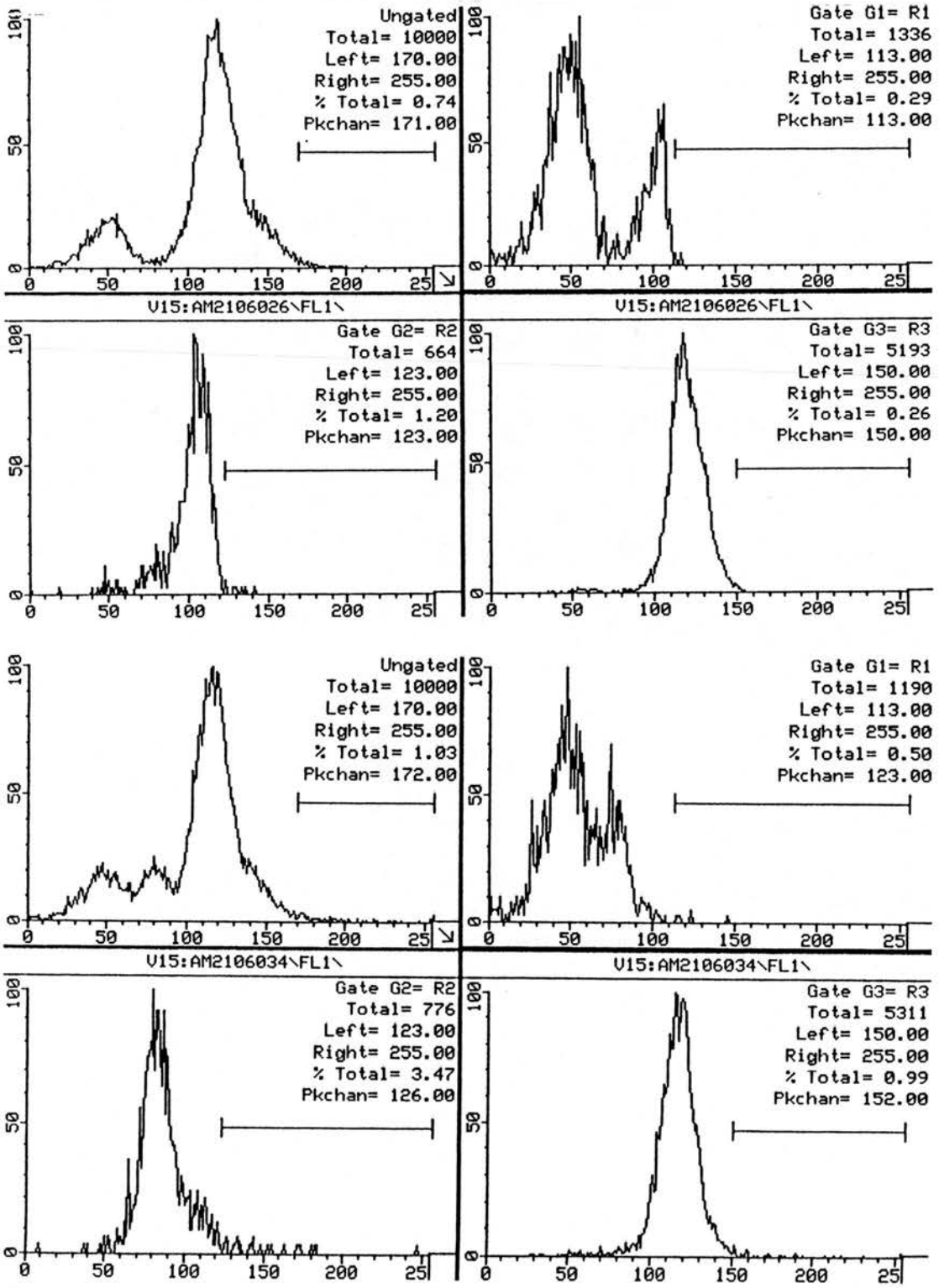

Ungated
Total= 10000
Left= 160.00
Right= 255.00
% Total= 3.03
Pkchan= 162.00
Gate G1= R1
Total= 1404
Left= 102.00
Right= 255.00
% Total= 2.35
Pkchan= 108.00
V13:AM2106032\FL1\
V13:AM2106032\FL1\
Gate G2= R2
Total= 840
Left= 109.00
Right= 255.00
% Total= 7.02
Pkchan= 111.00
Gate G3= R3
Total= 4978
Left= 126.00
Right= 255.00
% Total= 6.50
Pkchan= 126.00
Ungated
Total= 10000
Left= 160.00
Right= 255.00
% Total= 0.50
Pkchan= 163.00
Gate G1= R1
Total= 1468
Left= 102.00
Right= 255.00
% Total= 0.34
Pkchan= 104.00
V13:AM2106024\FL1\
V13:AM2106024\FL1\
Gate G2= R2
Total= 766
Left= 109.00
Right= 255.00
% Total= 1.04
Pkchan= 112.00
Gate G3= R3
Total= 5149
Left= 126.00
Right= 255.00
% Total= 0.97
Pkchan= 126.00

Figure 6.7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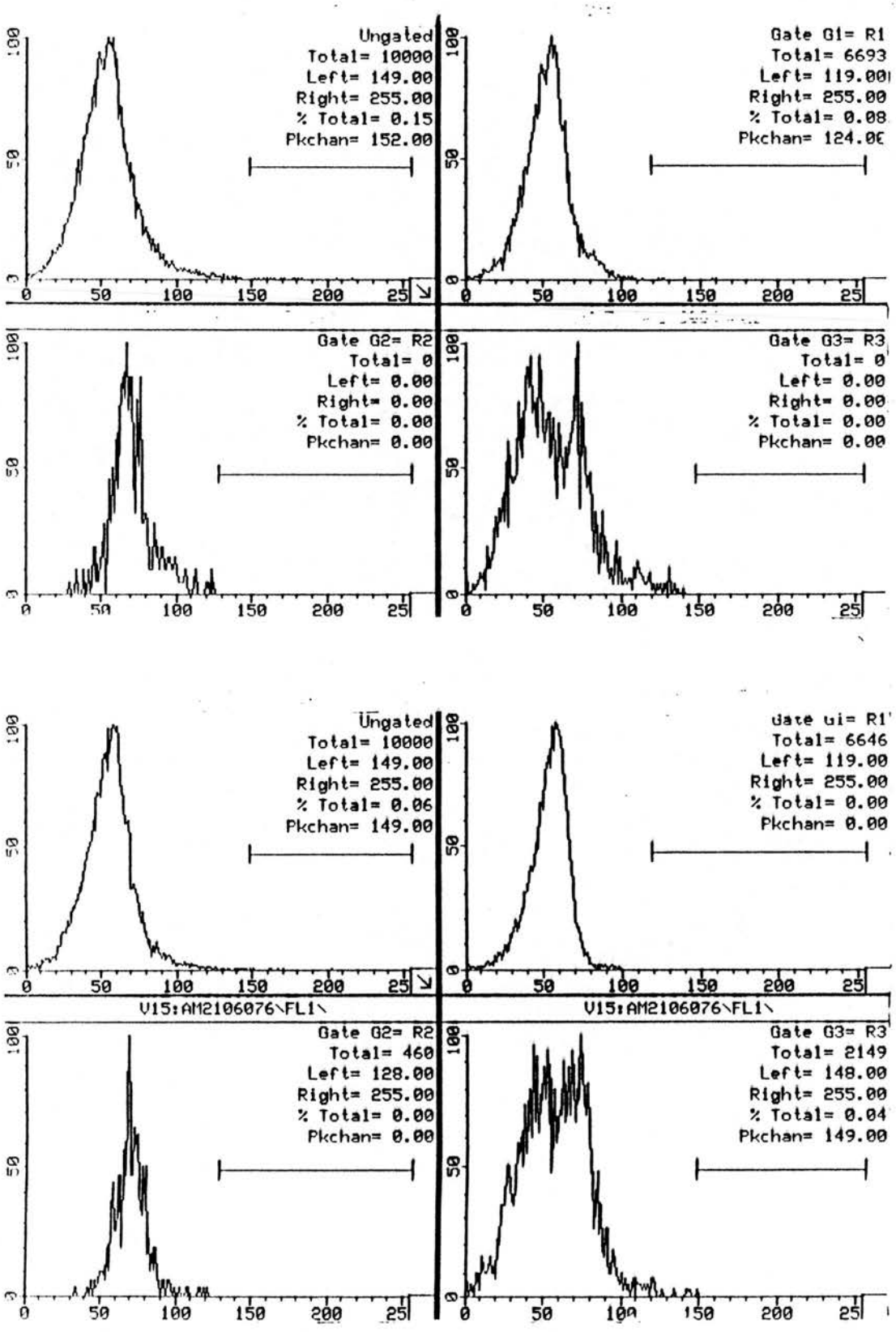
% Total= 0.74
Pkchan= 171.00
Gate G1= R1
Total= 1336
Left= 113.00
Right= 255.00
% Total= 0.29
Pkchan= 113.00
V15:AM2106026\FL1\
V15:AM2106026\FL1\
Gate G2= R2
Total= 664
Left= 123.00
Right= 255.00
% Total= 1.20
Pkchan= 123.00
Gate G3= R3
Total= 5193
Left= 150.00
Right= 255.00
% Total= 0.26
Pkchan= 150.00
Ungated
Total= 10000
Left= 170.00
Right= 255.00
% Total= 1.03
Pkchan= 172.00
Gate G1= R1
Total= 1190
Left= 113.00
Right= 255.00
% Total= 0.50
Pkchan= 123.00
V15:AM2106034\FL1\
V15:AM2106034\FL1\
Gate G2= R2
Total= 776
Left= 123.00
Right= 255.00
% Total= 3.47
Pkchan= 126.00
Gate G3= R3
Total= 5311
Left= 150.00
Right= 255.00
% Total= 0.99
Pkchan= 152.00

Figures 6.8, 6.9 and 6.10

Bone marrow cells (6.8) PBL (6.9) and peritoneal lavage cells (6.10) from a rat infected 5 days previously with *N. brasiliensis* and labelled with MARE-FITC,(top) or for control purposes, VPM-FITC (bottom) are analysed by flow cytometry. The parameters used for selecting the 3 gates and for determining fluorescence are those described in Figure 6.5

Figure 6.8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% Total= 0.15
Pkchan= 152.00
Gate G1= R1
Total= 6693
Left= 119.00
Right= 255.00
% Total= 0.08
Pkchan= 124.00
Gate G2= R2
Total= 0
Left= 0.00
Right= 0.00
% Total= 0.00
Pkchan= 0.00
Gate G3= R3
Total= 0
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Right= 0.00
% Total= 0.00
Pkchan= 0.00
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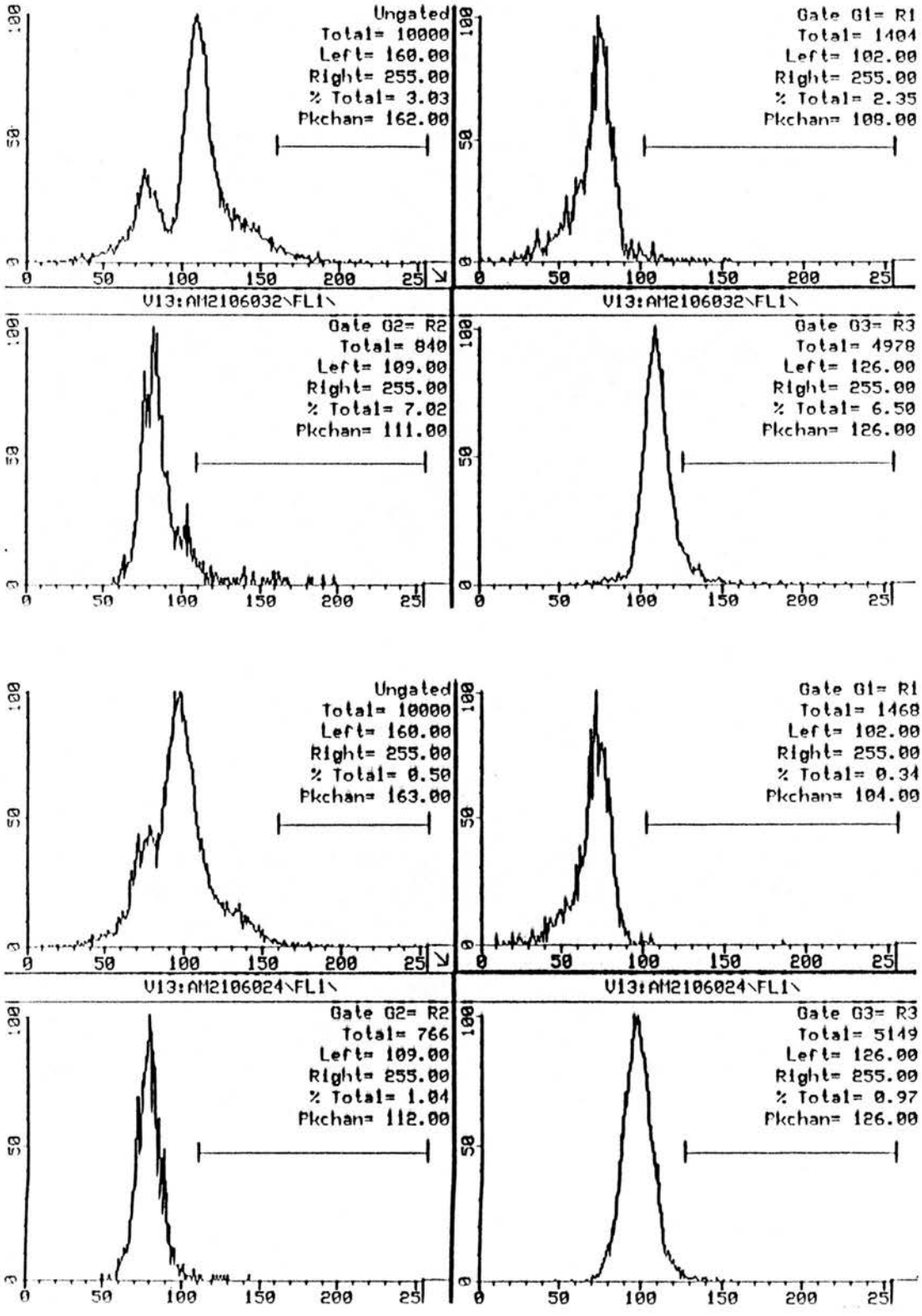
Left= 149.00
Right= 255.00
% Total= 0.06
Pkchan= 149.00
Gate G1= R1
Total= 6646
Left= 119.00
Right= 255.00
% Total= 0.00
Pkchan= 0.00
V15:AM2106076\FL1\
V15:AM2106076\FL1\
Gate G2= R2
Total= 460
Left= 128.00
Right= 255.00
% Total= 0.00
Pkchan= 0.00
Gate G3= R3
Total= 2149
Left= 148.00
Right= 255.00
% Total= 0.04
Pkchan= 149.00

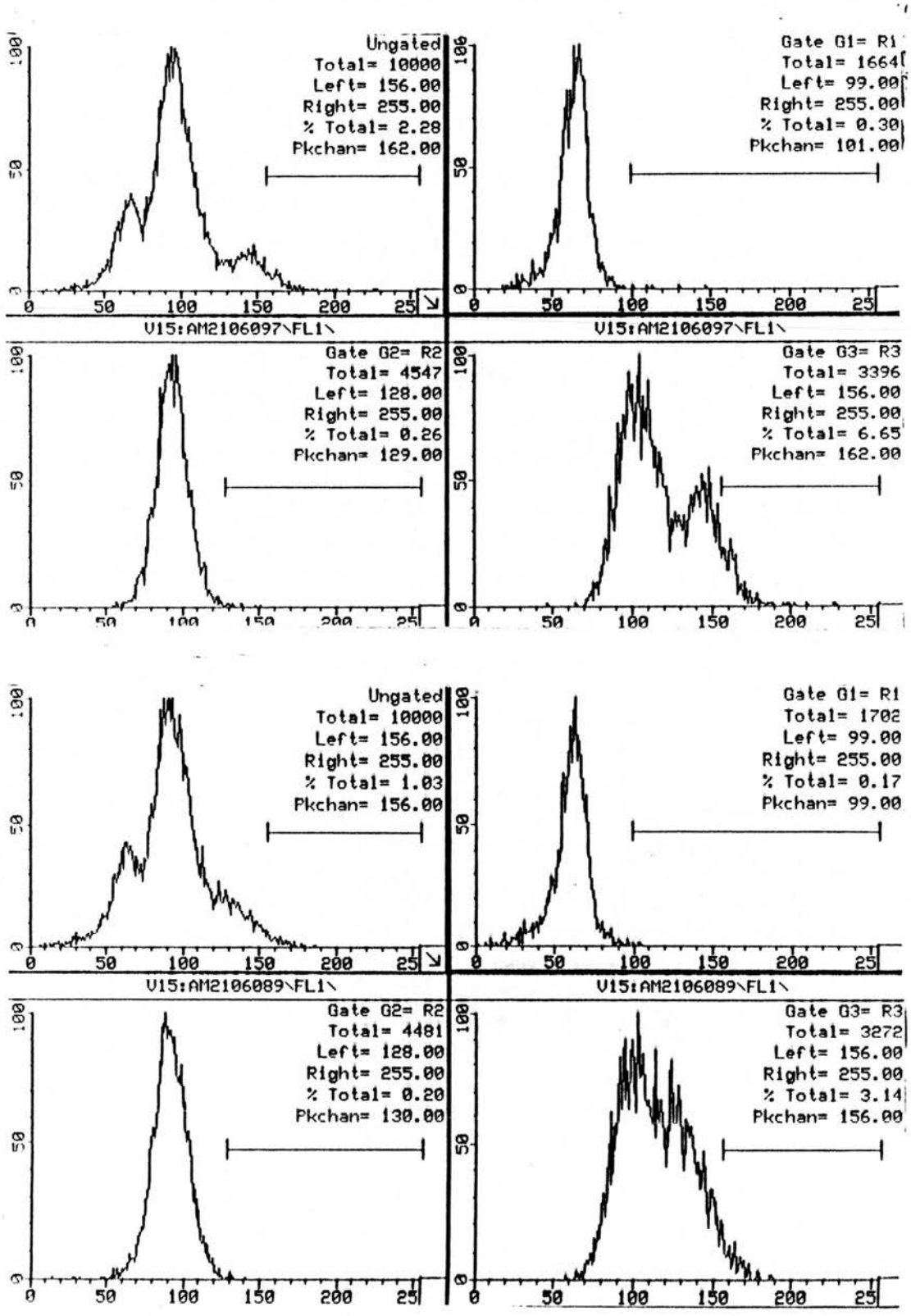
Figure 6.9


Ungated
Total= 10000
Left= 160.00
Right= 255.00
% Total= 3.03
Pkchan= 162.00
Gate G1= R1
Total= 1404
Left= 102.00
Right= 255.00
% Total= 2.35
Pkchan= 108.00
V13:AM2106032\FL1\
V13:AM2106032\FL1\
Gate G2= R2
Total= 840
Left= 109.00
Right= 255.00
% Total= 7.02
Pkchan= 111.00
Gate G3= R3
Total= 4978
Left= 126.00
Right= 255.00
% Total= 6.50
Pkchan= 126.00
Ungated
Total= 10000
Left= 160.00
Right= 255.00
% Total= 0.50
Pkchan= 163.00
Gate G1= R1
Total= 1468
Left= 102.00
Right= 255.00
% Total= 0.34
Pkchan= 104.00
V13:AM2106024\FL1\
V13:AM2106024\FL1\
Gate G2= R2
Total= 766
Left= 109.00
Right= 255.00
% Total= 1.04
Pkchan= 112.00
Gate G3= R3
Total= 5149
Left= 126.00
Right= 255.00
% Total= 0.97
Pkchan= 126.00

Day 10. The proportions of IgE-bearing cells in ungated BM ($P < 0.001$) and peritoneal lavage ($P < 0.001$), but not PBL ($P > 0.05$) were increased on day 10 of infection. The average frequency of labelled cells was 5.3%, 2.5% and 19% in BM, PBL and peritoneal lavage respectively (Fig. 6.4). There were two distinct populations in BM (Fig. 6.11), one with a high fluorescence intensity (Channels 180 to 210) composed almost exclusively of complex and large cells (high forward and high side scatter) which were presumably granulocytes and their precursors (Fig. 6.11, gate 3). The other had a low to intermediate intensity of fluorescence (Channels 130 to 180) composed of cells with medium FSC and low to medium SSC, presumably large lymphocytes and monocytes (Fig. 6.11, gate 1). Similar results were noted by fluorescence microscopy where two populations of cells were distinguished, large bright cells, and smaller cells with lower staining intensity.

Despite the increased proportion of fluorescent cells in the peritoneum ($P < 0.001$) (Fig. 6.4), there was no obvious single peak of fluorescence in any of the gates which might be indicative of a single, labelled, homogeneous population. Instead there was a range of cells with medium to high fluorescence intensity within all three gates (Fig. 6.13, gate 1; 4.6%, gate 2; 21% and gate 3; 46%). This was confirmed by UV-M where strongly fluorescent cells of variable size were detected. In particular, there were very large and bright cells which had a size-range and morphology similar to those of mast cells (not shown).

Figure 6.10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Total= 10000
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Right= 255.00
% Total= 2.28
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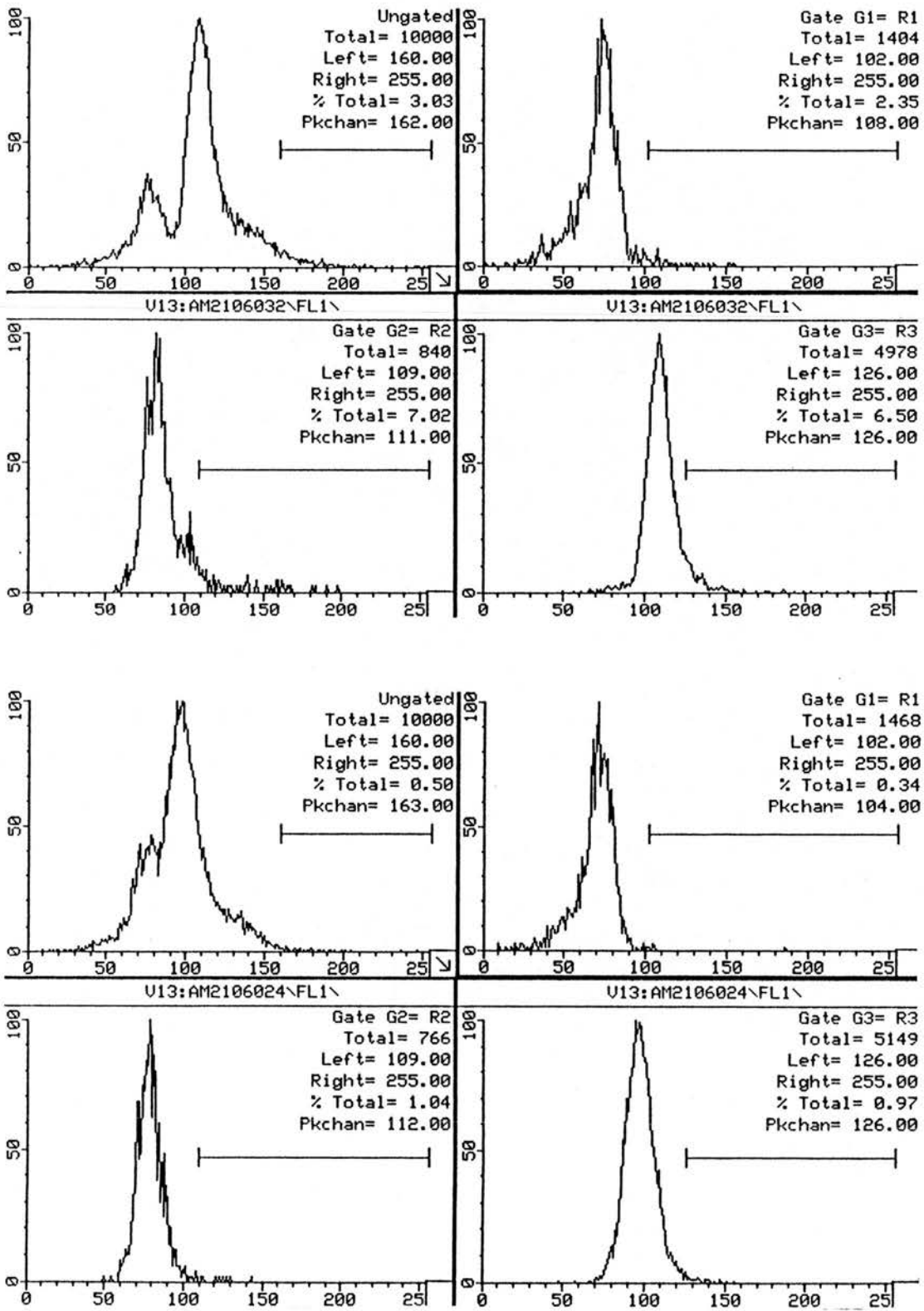
Gate G1= R1
Total= 1664
Left= 99.00
Right= 255.00
% Total= 0.30
Pkchan= 101.00
V15:AM2106097\FL1\
Gate G2= R2
Total= 4547
Left= 128.00
Right= 255.00
% Total= 0.26
Pkchan= 129.00
Gate G3= R3
Total= 3396
Left= 156.00
Right= 255.00
% Total= 6.65
Pkchan= 162.00
Ungated
Total= 10000
Left= 156.00
Right= 255.00
% Total= 1.03
Pkchan= 156.00
V15:AM2106089\FL1\
Gate G1= R1
Total= 1702
Left= 99.00
Right= 255.00
% Total= 0.17
Pkchan= 99.00
V15:AM2106089\FL1\
Gate G2= R2
Total= 4481
Left= 128.00
Right= 255.00
% Total= 0.20
Pkchan= 130.00
Gate G3= R3
Total= 3272
Left= 156.00
Right= 255.00
% Total= 3.14
Pkchan= 156.00

Figure 6.11, 6.12 and 6.13

Bone marrow (6.11) PBL (6.12) and peritoneal lavage cells (6.13) from rats infected 10 days previously with *N. brasiliensis* and labelled with MARE-FITC (top) and VPM-FITC (bottom) are analysed by flow cytometry.

Figure 6.11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Pkchan= 162.00
Gate G1= R1
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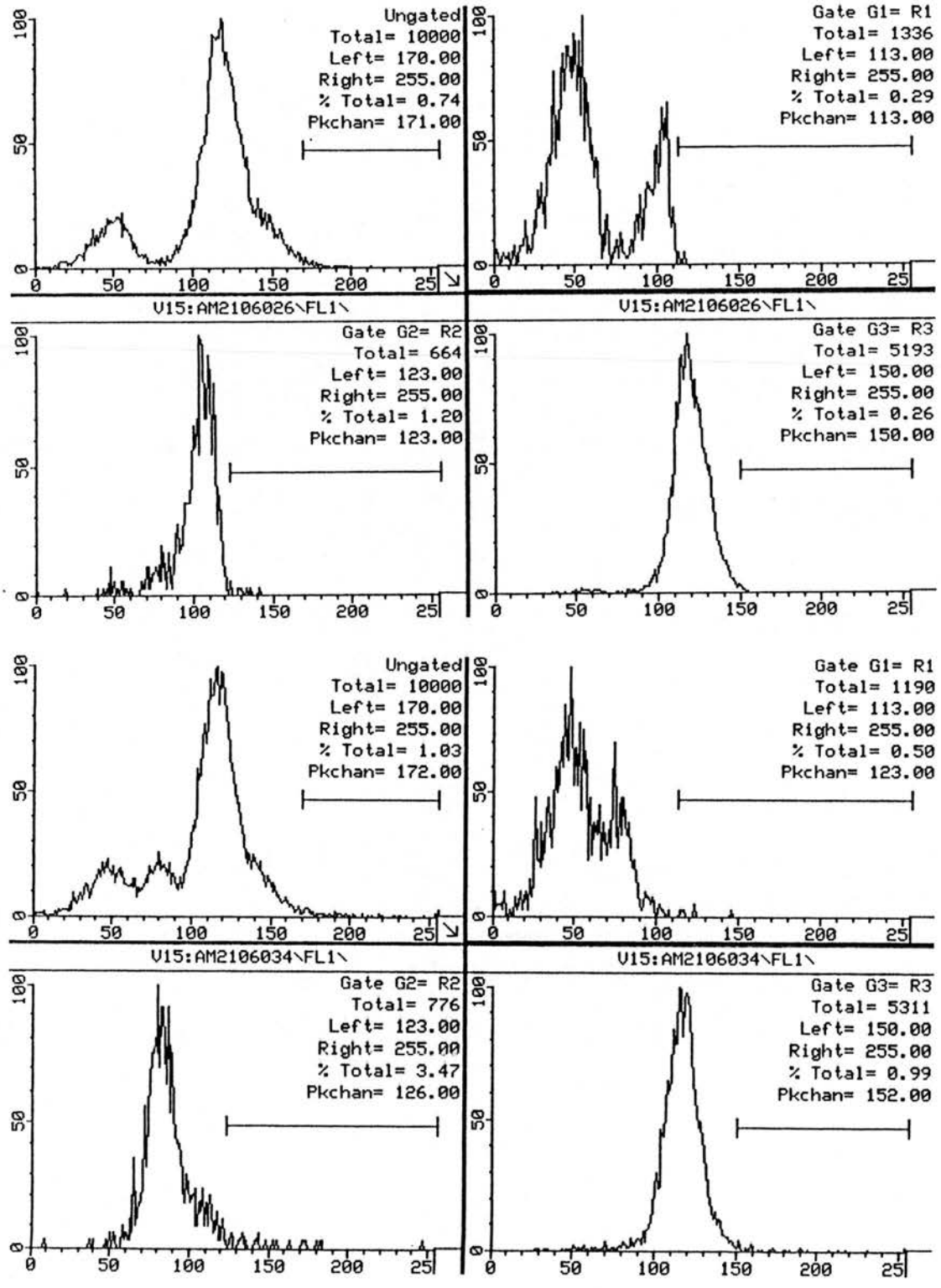
Left= 102.00
Right= 255.00
% Total= 2.35
Pkchan= 108.00
V13:AM2106032\FL1\
V13:AM2106032\FL1\
Gate G2= R2
Total= 840
Left= 109.00
Right= 255.00
% Total= 7.02
Pkchan= 111.00
Gate G3= R3
Total= 4978
Left= 126.00
Right= 255.00
% Total= 6.50
Pkchan= 126.00
Ungated
Total= 10000
Left= 160.00
Right= 255.00
% Total= 0.50
Pkchan= 163.00
Gate G1= R1
Total= 1468
Left= 102.00
Right= 255.00
% Total= 0.34
Pkchan= 104.00
V13:AM2106024\FL1\
V13:AM2106024\FL1\
Gate G2= R2
Total= 766
Left= 109.00
Right= 255.00
% Total= 1.04
Pkchan= 112.00
Gate G3= R3
Total= 5149
Left= 126.00
Right= 255.00
% Total= 0.97
Pkchan= 126.00

Figure 6.12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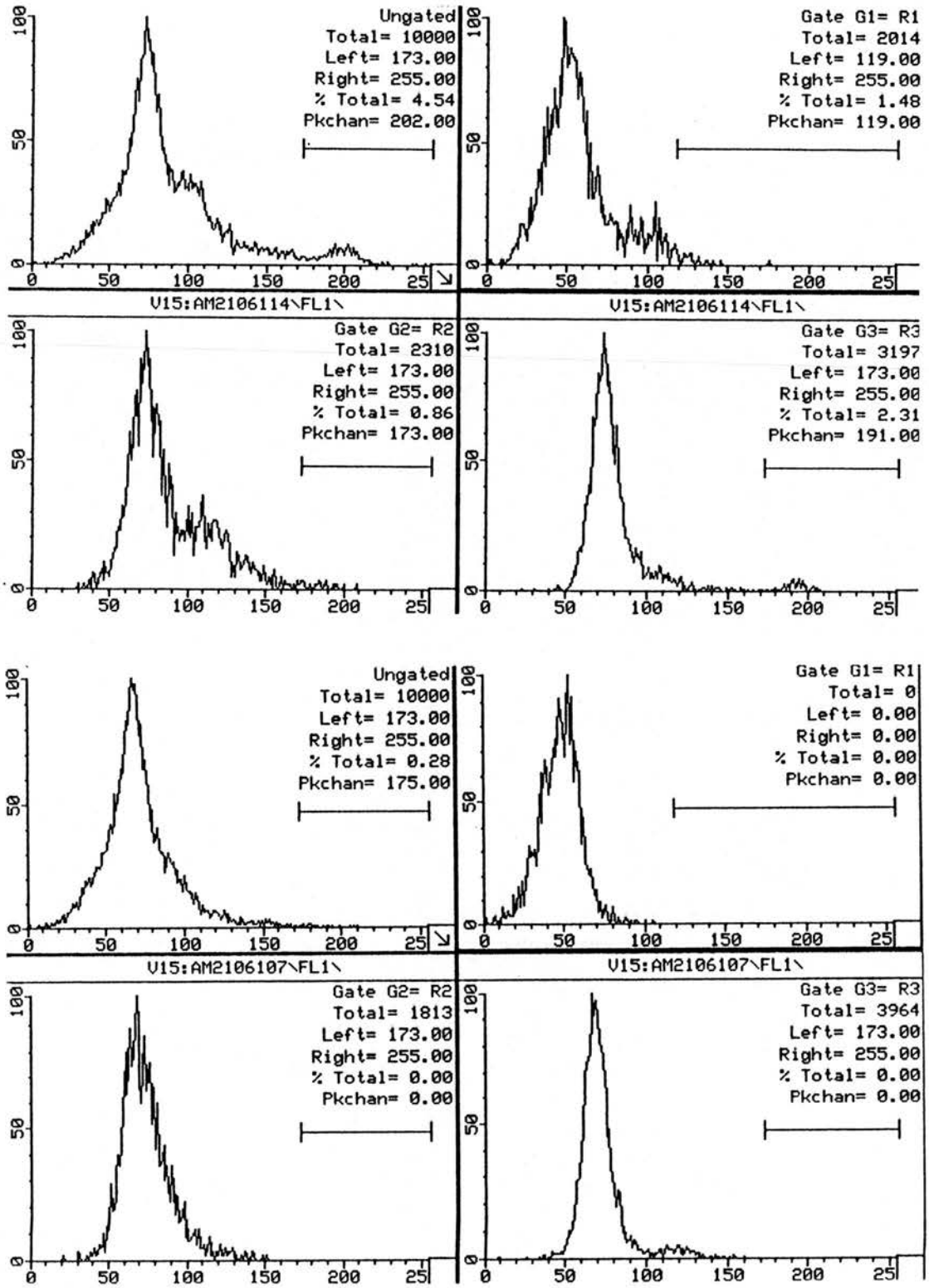
% Total= 0.74
Pkchan= 171.00
V15:AM2106026\FL1\
Gate G1= R1
Total= 1336
Left= 113.00
Right= 255.00
% Total= 0.29
Pkchan= 113.00
V15:AM2106026\FL1\
Gate G2= R2
Total= 664
Left= 123.00
Right= 255.00
% Total= 1.20
Pkchan= 123.00
Gate G3= R3
Total= 5193
Left= 150.00
Right= 255.00
% Total= 0.26
Pkchan= 150.00
Ungated
Total= 10000
Left= 170.00
Right= 255.00
% Total= 1.03
Pkchan= 172.00
V15:AM2106034\FL1\
Gate G1= R1
Total= 1190
Left= 113.00
Right= 255.00
% Total= 0.50
Pkchan= 123.00
V15:AM2106034\FL1\
Gate G2= R2
Total= 776
Left= 123.00
Right= 255.00
% Total= 3.47
Pkchan= 126.00
Gate G3= R3
Total= 5311
Left= 150.00
Right= 255.00
% Total= 0.99
Pkchan= 152.00

Figure 6.13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% Total= 4.54
Pkchan= 202.00
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Gate G1= R1
Total= 2014
Left= 119.00
Right= 255.00
% Total= 1.48
Pkchan= 119.00
V15:AM2106114\FL1\
Gate G2= R2
Total= 2310
Left= 173.00
Right= 255.00
% Total= 0.86
Pkchan= 173.00
Gate G3= R3
Total= 3197
Left= 173.00
Right= 255.00
% Total= 2.31
Pkchan= 191.00
Ungated
Total= 10000
Left= 173.00
Right= 255.00
% Total= 0.28
Pkchan= 175.00
V15:AM2106107\FL1\
Gate G1= R1
Total= 0
Left= 0.00
Right= 0.00
% Total= 0.00
Pkchan= 0.00
V15:AM2106107\FL1\
Gate G2= R2
Total= 1813
Left= 173.00
Right= 255.00
% Total= 0.00
Pkchan= 0.00
Gate G3= R3
Total= 3964
Left= 173.00
Right= 255.00
% Total= 0.00
Pkchan= 0.00

Day 15 The FACScan profiles of BM cells harvested on day 15 of infection were similar to those on day 10 (Figure 6.14), despite the significant ($P < 0.001$) reduction in the proportion of IgE-bearing cells on day 15 (Fig. 6.4). This was confirmed by UV-M. Nonetheless, two distinct populations of labelled cells were identified, large complex cells and large, less complex cells which expressed high (Channels 180 to 220), and low to intermediate, intensity of fluorescence (Channels 100 to 130), respectively (not shown).

The proportions of IgE-bearing cells in PBL had increased to 29% ($P < 0.001$) (Fig. 6.4) and two distinct cell populations could be distinguished by FACScan. The larger of the two, (Channels 100 to 180) was composed predominantly of cells with low foward and side scatter (Fig. 6.15, gate 2). A much smaller peak with greater intensity of fluorescence (Channels 190 to 210) comprising complex large cells with high side and high forward scatter was also present (Fig. 6.15, Gate 4). Large numbers of labelled cells were also noted by UV-M and their size was comparable to that of lymphocytes.

Sixteen percent of the peritoneal cells harvested on day 15 were positive for surface IgE by FACScan (Fig. 6.4) which was a significant reduction when compared with values on day 10 ($P < 0.001$). Almost all labelled cells had high foward and side scatter (Fig. 6.16). Under the microscope the fluorescent cells were similar in size and morphology to mast cells.

Figure 6.14

Bone marrow cells (6.14) PBL (6.15) and peritoneal lavage cells (6.16) from a rat infected 15 days previously with *N. brasiliensis* and labelled with MARE-FITC,(top) or for control purposes, VPM-FITC (bottom) are analysed by flow cytometry. The parameters used for selecting the 3 gates and for determining fluorescence are those described in Figure 6.5

Figure 6.14

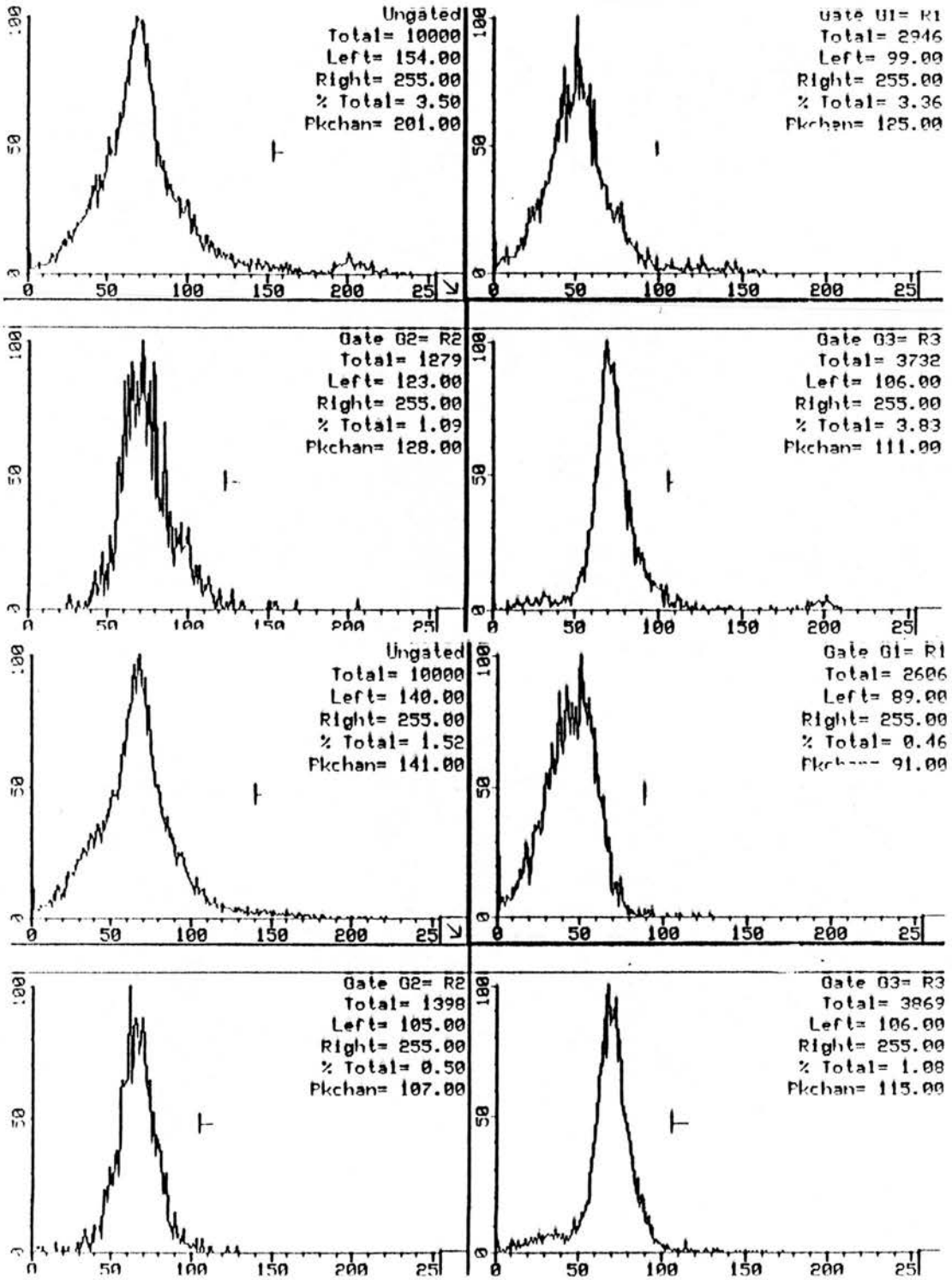
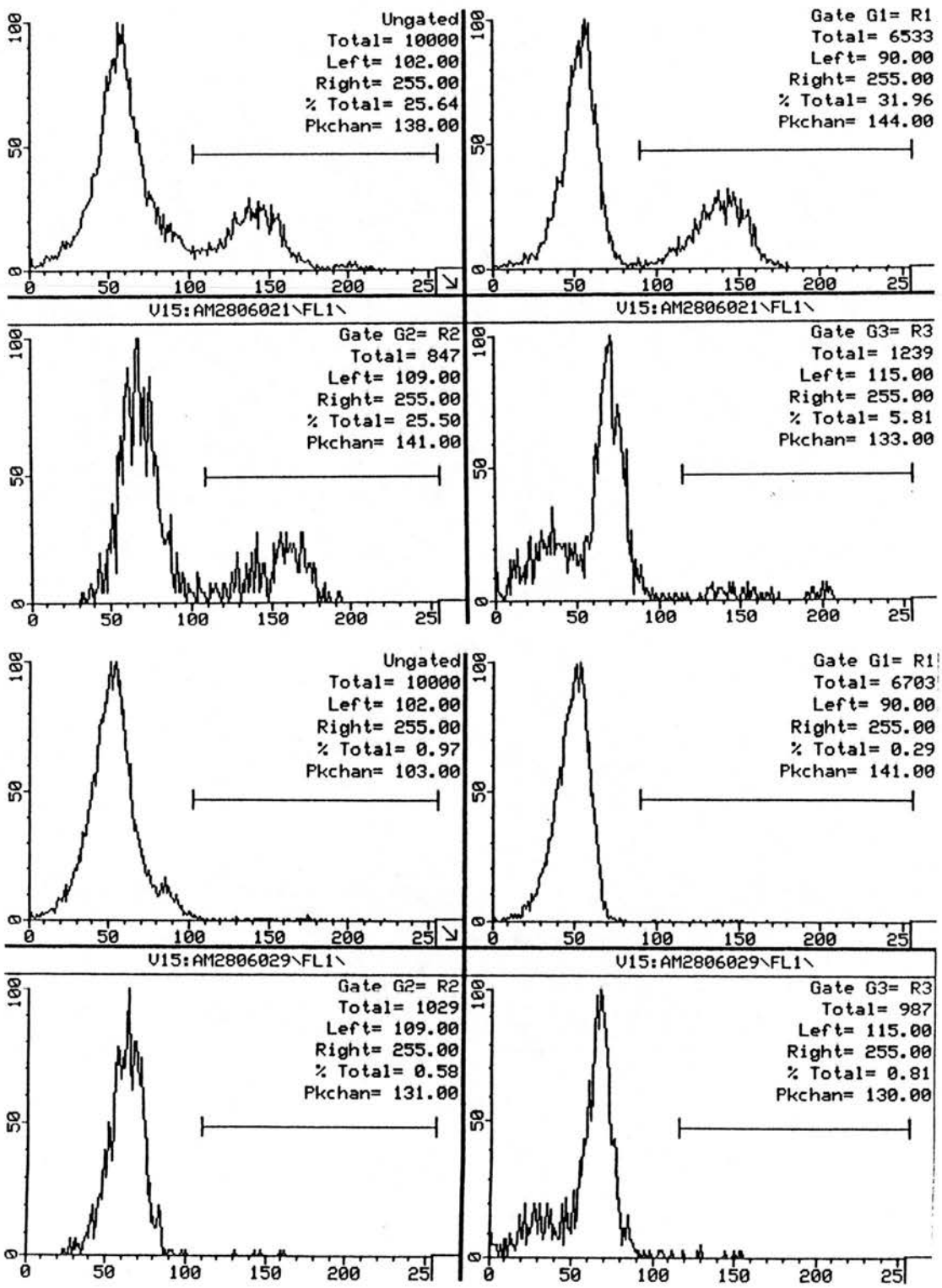

Ungated
Total= 10000
Left= 154.00
Right= 255.00
% Total= 3.50
Pkchan= 201.00
Gate G1= R1
Total= 2946
Left= 99.00
Right= 255.00
% Total= 3.36
Pkchan= 125.00
Gate G2= R2
Total= 1279
Left= 123.00
Right= 255.00
% Total= 1.09
Pkchan= 128.00
Gate G3= R3
Total= 3732
Left= 106.00
Right= 255.00
% Total= 3.83
Pkchan= 111.00
Ungated
Total= 10000
Left= 140.00
Right= 255.00
% Total= 1.52
Pkchan= 141.00
Gate G1= R1
Total= 2606
Left= 89.00
Right= 255.00
% Total= 0.46
Pkchan= 91.00
Gate G2= R2
Total= 1398
Left= 105.00
Right= 255.00
% Total= 0.50
Pkchan= 107.00
Gate G3= R3
Total= 3869
Left= 106.00
Right= 255.00
% Total= 1.08
Pkchan= 115.00

Figure 6.15


Ungated
Total= 10000
Left= 102.00
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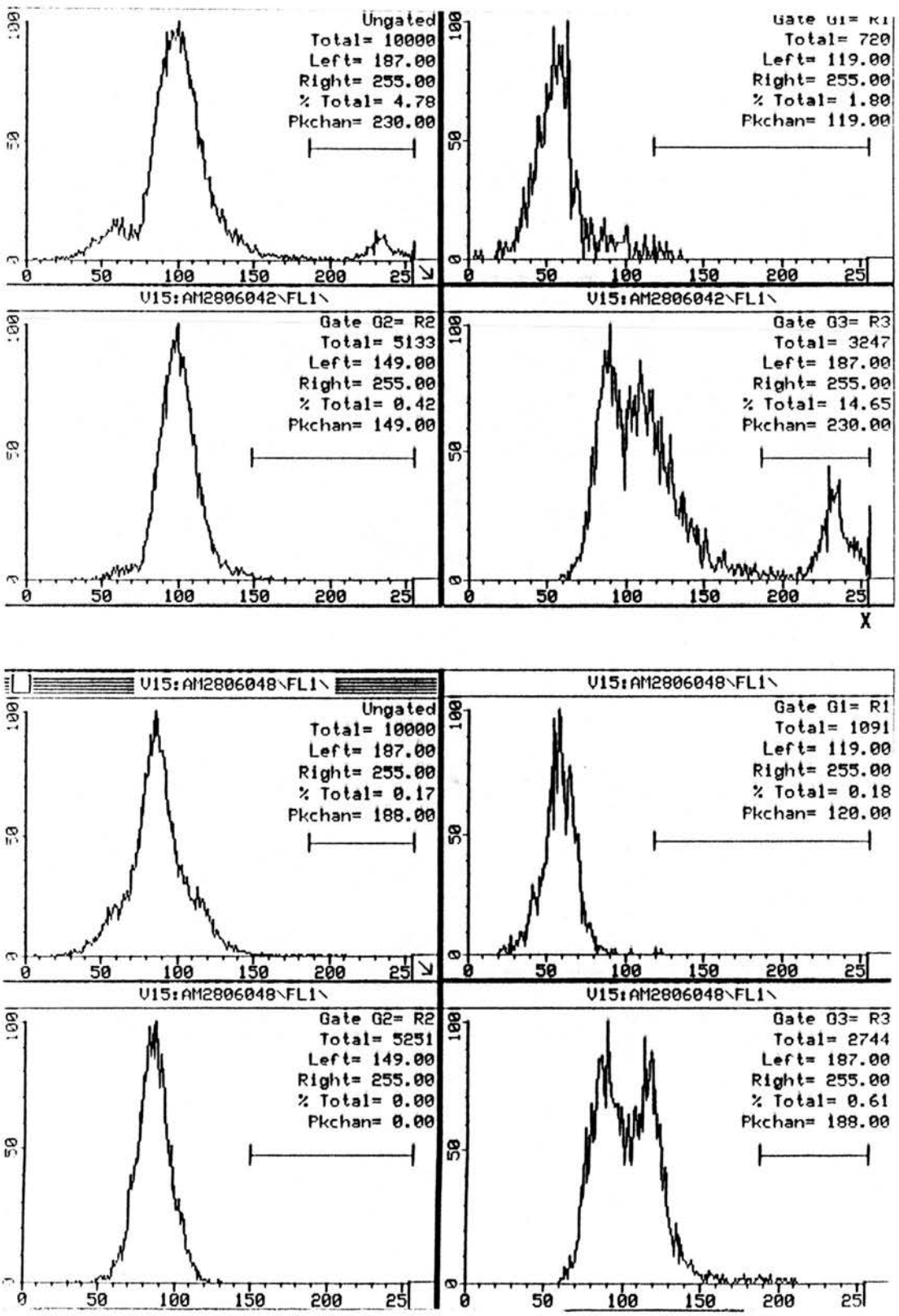
% Total= 25.64
Pkchan= 138.00
Gate G1= R1
Total= 6533
Left= 90.00
Right= 255.00
% Total= 31.96
Pkchan= 144.00
V15:AM2806021\FL1\
V15:AM2806021\FL1\
Gate G2= R2
Total= 847
Left= 109.00
Right= 255.00
% Total= 25.50
Pkchan= 141.00
Gate G3= R3
Total= 1239
Left= 115.00
Right= 255.00
% Total= 5.81
Pkchan= 133.00
Ungated
Total= 10000
Left= 102.00
Right= 255.00
% Total= 0.97
Pkchan= 103.00
Gate G1= R1
Total= 6703
Left= 90.00
Right= 255.00
% Total= 0.29
Pkchan= 141.00
V15:AM2806029\FL1\
V15:AM2806029\FL1\
Gate G2= R2
Total= 1029
Left= 109.00
Right= 255.00
% Total= 0.58
Pkchan= 131.00
Gate G3= R3
Total= 987
Left= 115.00
Right= 255.00
% Total= 0.81
Pkchan= 130.00

Figure 6.16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% Total= 4.78
Pkchan= 230.00
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Total= 720
Left= 119.00
Right= 255.00
% Total= 1.80
Pkchan= 119.00
V15:AM2806042\FL1\
Gate G2= R2
Total= 5133
Left= 149.00
Right= 255.00
% Total= 0.42
Pkchan= 149.00
V15:AM2806042\FL1\
Gate G3= R3
Total= 3247
Left= 187.00
Right= 255.00
% Total= 14.65
Pkchan= 230.00
X
V15:AM2806048\FL1\
Ungated
Total= 10000
Left= 187.00
Right= 255.00
% Total= 0.17
Pkchan= 188.00
V15:AM2806048\FL1\
Gate G1= R1
Total= 1091
Left= 119.00
Right= 255.00
% Total= 0.18
Pkchan= 120.00
V15:AM2806048\FL1\
Gate G2= R2
Total= 5251
Left= 149.00
Right= 255.00
% Total= 0.00
Pkchan= 0.00
V15:AM2806048\FL1\
Gate G3= R3
Total= 2744
Left= 187.00
Right= 255.00
% Total= 0.61
Pkchan= 188.00

Day 25 There was a small reduction in the proportion of IgE-bearing cells in bone marrow when compared with day 15 but it was not statistically significant ($P > 0.05$) and the fluorescence profiles on both days were very similar. Two populations of cells were noted, an intensely fluorescent population (Channels 190 to 210) with high foward and side scatter and a lightly labelled population (Channel 100 to 130). Only small numbers of IgE-positive cells were demonstrable by microscopy and these had typical mast cell morphology and size.

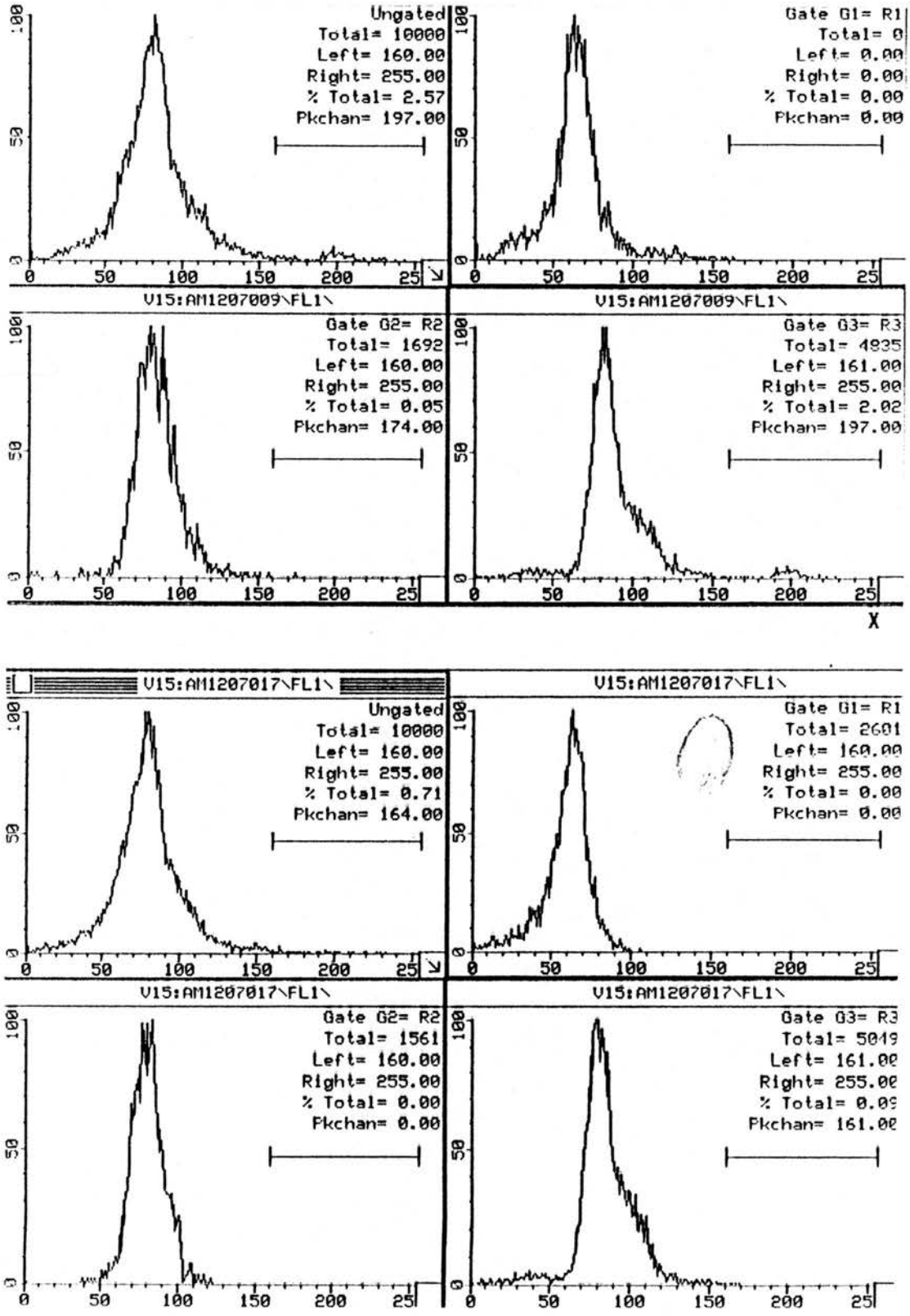
The proportion of IgE-bearing cells in peripheral blood was also reduced to 16% ($P < 0.001$). The majority had low to intermediate fluorescence intensity (Channels 100 to 150). The population with high fluorescent intensity (Channels 190 to 210) noted on day 15 had all but disappeared. Despite this, IgE-bearing cells were still demonstrable within all three gates, particularly in gates 1 and 2. As noted on day 15, many labelled cells were small lymphocyte-like.

Although the proportions of IgE-bearing cells in peritoneal lavage increased to 14% on day 25 (Fig. 6.4) this was not significant ($P < 0.05$). There were two distinct populations of labelled cells:- a shoulder on the main peak which had low to intermediate intensity of fluorescence (Channels 160 to 200) and high forward and low side scatter, presumably monocytes and large lymphocytes and a high intensity peak which accounts for just over 1/5 of the labelled cells in this compartment. The latter were identified as mast cells by UV-M and light microscopy.

Figure 6.17

Bone marrow cells (6.17) PBL (6.18) and peritoneal lavage cells (6.19) from a rat infected 25 days previously with *N. brasiliensis* and labelled with MARE-FITC,(top) or for control purposes, VPM-FITC (bottom) are analysed by flow cytometry. The parameters used for selecting the 3 gates and for determining fluorescence are those described in Figure 6.5

Figure 6.17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Right= 255.00
% Total= 2.57
Pkchan= 197.00
Gate G1= R1
Total= 0
Left= 0.00
Right= 0.00
% Total= 0.00
Pkchan= 0.00
V15:AM1207009\FL1\
Gate G2= R2
Total= 1692
Left= 160.00
Right= 255.00
% Total= 0.05
Pkchan= 174.00
Gate G3= R3
Total= 4935
Left= 161.00
Right= 255.00
% Total= 2.02
Pkchan= 197.00
V15:AM1207017\FL1\
Ungated
Total= 10000
Left= 160.00
Right= 255.00
% Total= 0.71
Pkchan= 164.00
Gate G1= R1
Total= 2601
Left= 160.00
Right= 255.00
% Total= 0.00
Pkchan= 0.00
Gate G2= R2
Total= 1561
Left= 160.00
Right= 255.00
% Total= 0.00
Pkchan= 0.00
Gate G3= R3
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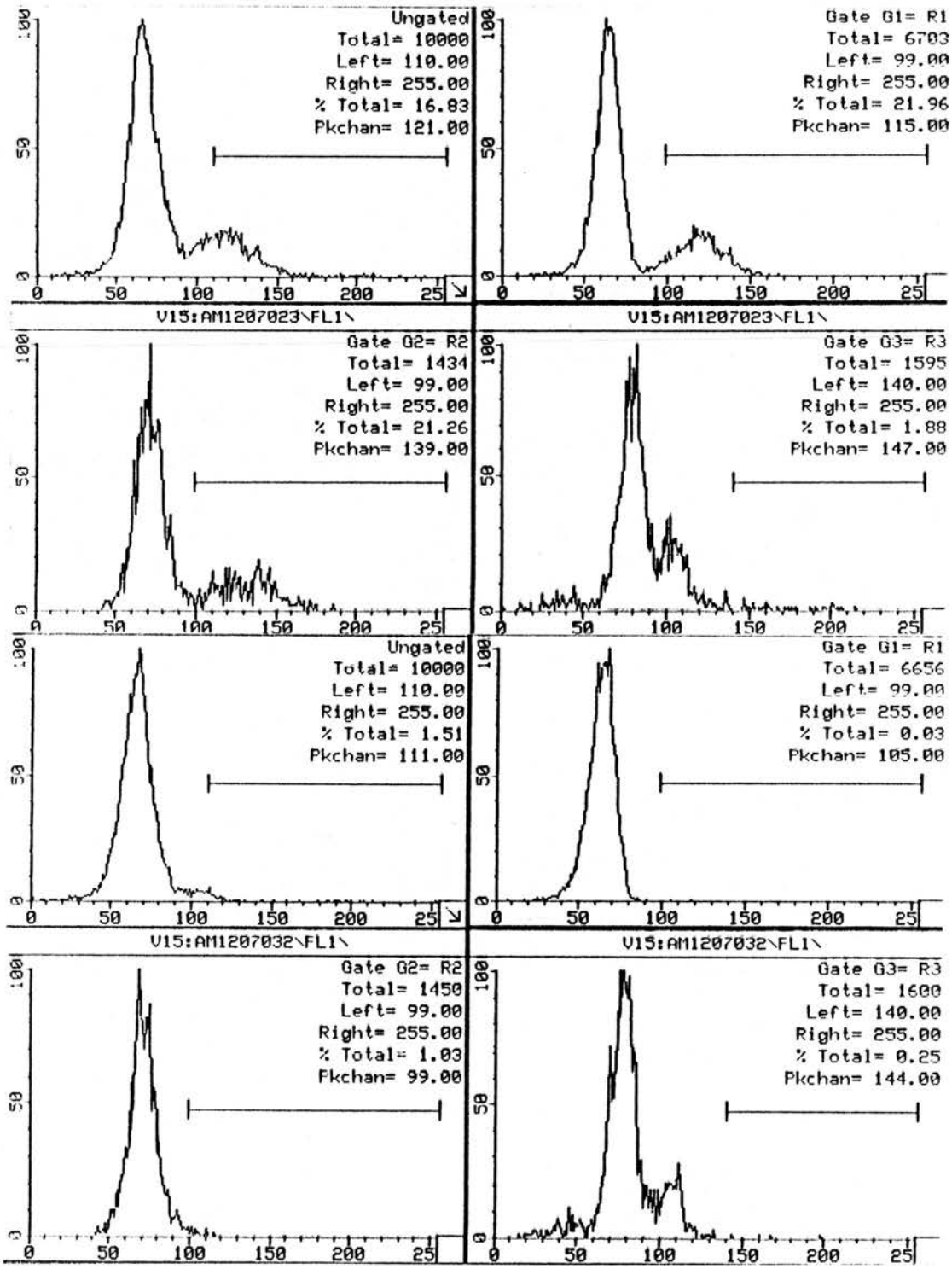
Right= 255.00
% Total= 0.09
Pkchan= 161.00

Figure 6.18


Ungated
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% Total= 16.83
Pkchan= 121.00
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Total= 6703
Left= 99.00
Right= 255.00
% Total= 21.96
Pkchan= 115.00
V15:AM1207023\FL1\
Gate G2= R2
Total= 1434
Left= 99.00
Right= 255.00
% Total= 21.26
Pkchan= 139.00
Gate G3= R3
Total= 1595
Left= 140.00
Right= 255.00
% Total= 1.88
Pkchan= 147.00
Ungated
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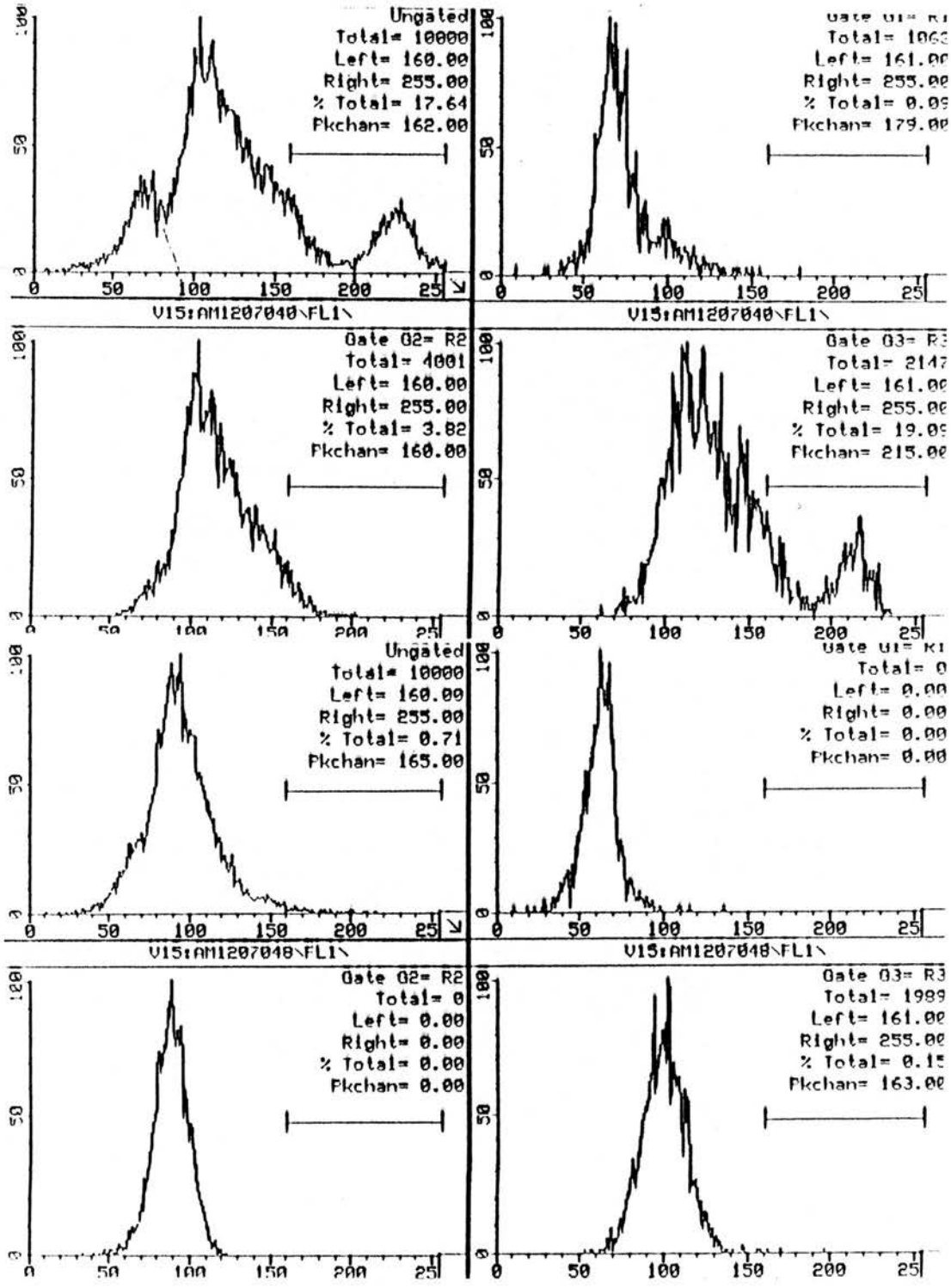
Left= 110.00
Right= 255.00
% Total= 1.51
Pkchan= 111.00
Gate G1= R1
Total= 6656
Left= 99.00
Right= 255.00
% Total= 0.03
Pkchan= 105.00
V15:AM1207032\FL1\
Gate G2= R2
Total= 1450
Left= 99.00
Right= 255.00
% Total= 1.03
Pkchan= 99.00
Gate G3= R3
Total= 1600
Left= 140.00
Right= 255.00
% Total= 0.25
Pkchan= 144.00

Figure 6.19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% Total= 17.64
Pkchan= 162.00
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Gate G2= R2
Total= 4001
Left= 160.00
Right= 255.00
% Total= 3.82
Pkchan= 160.00
Gate G3= R3
Total= 2147
Left= 161.00
Right= 255.00
% Total= 19.09
Pkchan= 215.00
Ungated
Total= 10000
Left= 160.00
Right= 255.00
% Total= 0.71
Pkchan= 165.00
V15:AM1207048\FL1\
Gate G2= R2
Total= 0
Left= 0.00
Right= 0.00
% Total= 0.00
Pkchan= 0.00
Gate G3= R3
Total= 1989
Left= 161.00
Right= 255.00
% Total= 0.15
Pkchan= 163.00

DISCUSSION

The kinetics of appearance of IgE-bearing, presumably $Fc_{\varepsilon}R$, positive cells was examined in 3 compartments. Bone marrow was chosen because it is the major source of haemopoietic cells and it is from this tissue that the progenitors of mast cells, eosinophils, monocytes and B cells arise (Haig and Miller, 1990). All of these cells will, with the appropriate stimulus, express $Fc_{\varepsilon}R$ and, since they are recruited to tissues via the bloodstream, it was important to determine whether this was reflected by changes in the proportions of $Fc_{\varepsilon}R$ cells in peripheral blood during the course of infection. Cells from the peritoneal cavity were examined because the population is known to fluctuate during infection (Smith and Bryant, 1989) and because early sensitization of mast cells in the peritoneal cavity has been reported (Wilson and Bloch, 1968).

Although cell sorting and phenotypic analysis was not conducted here, it is not unreasonable to make general conclusions about phenotype, based on previous studies. Lymphocytes have low forward and side scatter and can be found in gate 1. Granulocytes and mast cells are much larger and more complex and found in gate 3. Gate 2 is composed predominantly of myeloid cells and large lymphocytes.

As already discussed in the introduction a variety of cell types express receptors for IgE. Lymphocytes (B and T) mast cells, eosinophils, basophils, macrophage may all express IgE on their cell surface. MARE-1-FITC is an IgG_1 isotype antibody and although it is a murine monoclonal, it may still bind to the rat receptor for rat IgG_1. To act as a control, VPM-FITC was chosen because it is the same murine isotype but has an irrelevant specificity.

Peritoneal lavage was the most interesting compartment, with two separate populations of FITC-stained on day 0. The population staining less intensely, was traced to gates 1 and 2 and is therefore likely to be myeloid and lymphoid cells predominantly. The larger population which stained more intensively was traced to gate 3 and is therefore likely to contain granulocytes and mast cells. Granulocytes characteristically express receptors for IgG and it is likely that there is some degree of association between the murine IgG_1 isotypes used in this analysis and the rat Fc receptors on cells in this compartment. The results from this study indicate that Fc binding of IgG_1 occurred in PBL and peritoneal lavage cells. During the time course the extent of Fc binding varied depending on the proportion of different cells at that time.

IgE-bearing cells were first demonstrable in the peritoneal lavage on day 10, and were noted in all three gates. The number of IgE-bearing cells decreased on day 15 but increased once again on day 25. The majority of IgE bearing cells stained intensely and

were restricted to gate 3. It is likely that mast cells or their precursors make up a proportion of this population. Binding of the IgG_1 isotype by Fc receptor was still demonstrable at this time but is easily distinguished from IgE-bearing cells.

In PBL there was a similar interaction between the IgG_1 isotype and the Fc receptor for IgG but was much less pronounced. This is due mainly to the differences in predominant cell type and the total numbers of granulocytes noted in the PBL and expression of IgG Fc receptors. IgE-bearing cells were first recorded in PBL on day 15, when more than 25% of cells stained positive. IgE-bearing cells were predominantly from gates 1 and 2 and were therefore lymphoid and myeloid. The total number of IgE-bearing cells declined to 16% on day 25 but IgE-bearing cells were still demonstrable in both gates 1 and 2.

Because, in this study, the presence of surface IgE was the parameter measured, the detection of Fc R cells is highly dependent on the presence of increased concentrations of IgE to ensure saturation of Fc R. Therefore, the relative paucity of IgE-bearing cells on day 5 of infection probably reflects the very low concentration of serum IgE at this stage of infection (Jarrett *et al.*, 1976).

There was very little change in the BM when compared with PL or PBL. The most significant result in BM was the increase in IgE-bearing cells on day 10 of infection. This is a time when mast cell recruitment is in progress in Wistar rats (Nawa and Miller,

1979). Analysis of the IgE-bearing cells by FACScan on day 10 revealed that the cells were distributed within gates 1, 2 and 3, suggesting that they were of varying size and complexity. It is possible that at least some of these cells were mast cell precursors although, without further, more detailed, analysis of their growth potential, of their immunohistochemistry (e.g. protease content) and of their biochemical properties, it is not possible to confirm their eventual fate. Katona (1984) also reported an increase in $Fc_{\varepsilon}R$ expression in rat BM cells collected after infection with *N. brasiliensis* and Kinawa *et al.*, (1990) observed a similar increase in Fc R expression in BM following intravenous injection of purified IgE. Eosinophils, monocytes/macrophage, lymphocytes, and basophils and mast cells can express $Fc_{\varepsilon}R$ (See introduction to the Chapter). This is consistent with the general increase in $Fc_{\varepsilon}R$ expression, observed in gates 1, 2 and 3 by FACScan noted in this Chapter. Furthermore, IgE-bearing cells were heterogeneous by UV-M and it would appear from this analysis that they were predominantly small mononuclear cells, granulocytes and mast cells. Non-specific or RFC-conjugate interactions were not apparent at any time in the BM.

It is, perhaps, not surprising that there was no comparable rise in the proportion of $Fc_{\varepsilon}R$ cells in the blood on day 10. The dynamics of recruitment of cell populations from bone marrow to tissues during inflammation is not easy to determine because at least 3 parameters must be known:- (i) The rate of release of cells from bone marrow into blood, (ii) The duration of their presence in the vasculature. (iii) the rate of recruitment in inflamed

tissues. So rapid is the accumulation of mast cells in the gut during infection with *N. brasiliensis* (Nawa and Miller, 1979), that it is reasonable to hypothesize that recruitment into tissues may be equal to, or exceed, the rate of release of progenitors into the blood. If this were to be the case, then there may be little or no obvious fluctuation in the proportion of IgE-bearing cells amongst PBL.. The apparent increase in Fc R positive cells in the peritoneum on day 10 in the absence of comparable increases amongst PBL supports this hypothesis. Nonetheless there was a neutrophilia, an increase in monocytes and a reduction in the number of circulating lymphocytes in the PBL population on day 10 as determined in Leishman-stained smears.

The proportion of IgE-bearing leukocytes in peripheral blood, rose from 2.5% on day 10 to almost 30% on day 15. At the same time the percentage of IgE-bearing cells in BM was reduced, which is consistent with the concept that cells are recruited from the BM to the periphery via the circulatory system. However, it is unlikely that the large proportion of IgE-bearing cells in PBL is derived from the relatively small proportion of IgE-bearing cells in BM (5.1%). As discussed already, the MLN increases greatly in size and, on day 15, more than 50% of the cells bear surface IgE (Urban *et al.*, 1984; Urban, Ishizaka and Bazin, 1980). The thoracic duct is known to transport large amounts of IgE as well as many IgE-secreting cells from the gut and mesenteric lymph node into the circulatory system (Allan and Mayrhofer, 1986). The authors noted a dramatic increase in the numbers of IgE-producing cells and in the concentration of IgE in lymph on day 15. It is

likely, therefore, that thoracic duct lymph is contributing to the increased numbers of IgE-bearing cells described here. The predominant IgE-bearing cell type in PBL was probably of lymphoid origin and this is consistent with earlier observations in MLN where 97% of IgE positive cells were lymphocytes (Urban *et al.*, 1980; Katona, Urban, Scher, Kanellopoulos-Langevia and Finkelman, 1982). Jarrett *et al.*, 1976 showed that the most significant increases in serum IgE concentrations in Hooded Lister rats occurred between days 6 and 8 of infection. Since IgE induces expression of its own receptor (Kinawa *et al.*, 1990; Katona, 1984; Manouvriez and Bazin, 1984), it is not surprising that cells in both the BM and peritoneal lavage carried significantly more cytophilic IgE, especially since, during infection with enteric nematodes such as *N. brasiliensis* and *T. spiralis*, the MLN increases 3 to 4 fold in size (Urban, Katona, Dean and Finkelman, 1984; Ishizaka, Urban and Ishizaka, 1980).

The regional lymph nodes, such as MLN and bronchial nodes, are the primary sites of IgE synthesis during infection with *N. brasiliensis* (Wanatabe and Kobayashi, 1988; Mayrhofer *et al.*, 1976; Allan and Mayrhofer, 1984). Befus *et al.*, (1982) proposed a model for IgE sequestration within the sites of IgE synthesis, such as bronchial lymph node and enteric tissues. This may explain why 19% of the cells in peritoneal lavage on day 10 carried surface IgE and only 5.1% in BM. However, the apparent differences in IgE-staining may also be explained by the different cell populations and maturity of cells in these two compartments.

Jarrett *et al* (1976) reported maximal concentrations of total IgE around day 15 of infection with *N. brasiliensis*, and, thereafter, levels declined. The proportions of IgE-bearing cells in BM and PBL declined after day 15, but this probably reflects a decline in $Fc_{\varepsilon}R$ expression since IgE titres remain raised above normal for several weeks after infection (Jarrett *et al.*, 1976).

The most obvious decrease in IgE-bearing cells occurred in the small lymphocyte compartment. As already discussed these are probably B cells derived from MLN and it is likely that the stimulation by the parasite, with the consequent production of cytokines such as IL-4 (Vercelli *et al.*, 1988) locally is responsible for their expression of Fc RII. The decline in number is presumably, therefore, related to the absence of parasites and reduced cytokine production rather than to any rapid decline in IgE titre.

In conclusion, therefore, the detection of IgE-bearing cells requires:- (i) The presence of sufficient IgE to sensitize cells expressing $Fc_{\varepsilon}RI$ and II. (ii) The induction of receptor expression during the activation or differentiation of the various cell populations. It would be interesting to compare the level of receptor expression in rats of early and late IgE responder status, using exogenous, *in vitro* sensitization, with IgE to identify Fc R bearing cells much earlier in the response when concentrations of serum IgE are low and to determine whether there is a correlation *in vivo* between IgE responsiveness and Fc R expression.

CHAPTER 7

GENERAL CONCLUSIONS/DISCUSSION

Passive cutaneous sensitization with homocytotropic antibodies has been used extensively for characterization of allergic responses and of allergens. The passive cutaneous anaphylaxis technique, in experimental animals, is semi-quantitative so that, unless individual allergens are isolated and tested, the full range of antibody specificities cannot be determined. For this reason Western blotting, which permits qualitative analysis, was used to identify allergens from somatic extracts (AWH) of adult *N. brasiliensis* worms. To this end, sera from five strains of rat (LOU, F344, Hooded Lister, August and Wistar) previously infected with *N. brasiliensis*, were tested by Western blotting and at least seven distinct allergens were repeatedly identified. This confirms the importance of the Western blotting technique as a qualitative assay for detection of parasite allergens.

Several of the allergens described in this thesis were identified for the first time. However, there were some similarities with previously published data. In particular, the low molecular weight allergens of 14,000 and 17,000 are probably similar to the allergens isolated by column chromatography (Petit *et al.*, 1980; Wilson *et al.*, 1967; Jones and Ogilvie, 1972). To further confirm the allergenicity of the 14,000 MW antigen detected by Western blotting it was electroeluted (together with 2 or 3 other components of similar molecular weight) and tested by PCA. The electroeluted proteins provoked strong reactions by PCA, confirming that an allergen was indeed present. It is also apparent that this electroeluted *N. brasiliensis* allergen retained its biological activity even after denaturation, SDS-treatment, and at least one freeze and thaw. Similarly, *N. brasiliensis* allergen

remained active by PCA after repeated freeze-thawing and prolonged storage at 4°C (Wilson, 1967). Fujita and Tsukidate (1982) noted that purified *D. immitis* allergen was resistant to proteases, periodate oxidation, and other physicochemical treatments. Some grass pollen allergens are also resistant to autoclaving or vigorous boiling (Augustin, 19591; Lowenstein *et al.*, 1987). The relative sensitivity of some allergens to denaturation following chemical or physical treatment, as discussed in the General Introduction, is therefore not cosmopolitan.

Recently Yamada *et al* (1991) employed the Western blotting technique to examine *N. brasiliensis* AWH and secreted ES allergens, but there were several differences from the present study. Yamada *et al* (1991) used Sprague-Dawley rats and conducted all analysis of excretory-secretory products under non-reducing conditions. As discussed in the General Introduction, some parasite allergens have a tendency to aggregate (Owhashi *et al.*, 1987a and b; Hussain *et al.*, 1973; Ambler *et al.*, 1972; 1973). It is, perhaps, not surprising, therefore, that different results were noted. Nonetheless 28,000 and 70,000 (69,000) MW allergens were demonstrable in both studies. However, Yamada *et al* (1991) failed to detect the low molecular weight allergens described previously where products secreted by *N. brasiliensis* were examined. For example, Hogarth-Scott (1967) identified low molecular weight allergenic components from *N. brasiliensis*, *T. canis* and *A. suum* by gel-filtration and PCA. By contrast, Yamada

et al (1991) described allergens of not less than 24,000 in preparations from *N. brasiliensis*. It is possible that under non-reducing conditions the low molecular weight *N. brasiliensis* allergens may associate non-specifically with larger proteins.

Future research should include comparative analysis of *N. brasiliensis* AWH and of excretory/secretory products under reducing and non-reducing conditions. From preliminary observations at this laboratory, there was very little difference between reduced and non-reduced *N. brasiliensis* AWH when fractionated by SDS-PAGE and stained with Coomassie blue stain, or when blotted and probed with immune serum and with polyvalent anti-immunoglobulins (results not shown). Also, for comparative purposes, the allergens recognized by Sprague-Dawley rats, after infection with *N. brasiliensis* L_3, should be defined under reducing and non-reducing conditions.

Parasites are complex organisms and contain a large number of proteins and potential immunogens. However it has been reported in a number of studies that infected animals failed to recognize all of the potential parasite antigens (Else and Wakelin, 1989; Kennedy, McIntosh, Blair and McLaughlin, 1990; Kennedy, Tomlinson, Fraser and Christie, 1990). It is widely acknowledged that both H-2-linked and background genes play important roles in controlling the humoral response to parasites in mice (Else and Wakelin, 1989; Wassom, Brooks and Cypess, 1983)

Recognition of some parasite antigens/allergens is restricted by the MHC complex; specifically, H-2 in mice, and RT1 in rats (Kennedy *et al.*, 1986, 1990; Tomlinson, Christie, Fraser,

McLaughlin, McIntosh and Kennedy, 1989). In contrast it was also noted that strains of mice with the same H-2 haplotype but with distinct genetic backgrounds may still recognize distinct antigens (Else and Wakelin, 1989). IgE like the other immunoglobulin isotypes is controlled by MHC-linked genes (Katz, 1980; Katz *et al.*, 1979; Tomlinson *et al.*, 1989). However, the magnitude of the IgE response is influenced by some non-MHC linked genes (Ishizaka, 1976; Bazin *et al.*, 1981; Ishizaka, 1981; Tomlinson *et al.*, 1989; Pfeiffer *et al.*, 1983). It is now established that the inability to synthesize specific IgE antibody, rather than being the result of genetic non-responsiveness, represents an ability to suppress the magnitude of the IgE response (Katz, 1980). In the present study all strains of rat tested produced IgE specific for the low MW allergens, however, F344 and, to a lesser extent, Wistar rats had reduced specific IgE responses until after secondary challenge. Since immunization with adjuvant can overcome the genetic controlled tendency to suppress IgE responses (Wakelin *et al.*, 1986), secondary infection with a large parasite burden may have had the same effect. The alternative possibility is that very low titres of parasite-specific IgE are produced and cannot be detected during primary infection.

Pfeiffer *et al*, (1983) measured titres of IgE antibody by PCA in 36 strains of mice infected with *N. brasiliensis*. Three response phenotyes were apparent, high responder, medium responder and low/non-responders. It was noted that concentrations of parasite-specific IgG_1 were greatest in high IgE responder strains. A similar association was described in *Ascaris* infection in mice (Tomlinson *et al.*, 1989). A 14,000 MW allergen from the body

fluids of *A. suum* is selectively recognized by strains with the H-2^s haplotype where specific IgE and IgG_1 responses were demonstrable but were both absent from non H-2^s haplotype-bearing strains (Tomlinson *et al.*, 1989).

The specificity of the IgE response was shown to vary between rat strains (Chapter 4). For example, all but F344 rats responded after primary infection and of the strains responding all but Wistar rats recognized the low molecular weight allergens (14,000 and 17,000). LOU rats were unique, responding to both high and low molecular weight allergens after primary infection. This is not the first report of variability between rat strains. Pfeiffer, Konig and Bohn (1983) used the PCA test to demonstrate high, low and non-responder IgE and IgG_1 phenotypes in mice infected with *N. brasiliensis*. Kennedy, McIntosh, Blair and McLaughlin (1990) characterized quantitative differences in the humoral responses of different rat strains infected with *N. brasiliensis* by Western blotting.

Variability was also noted within rat strains; Wistar rats responded to six distinct high molecular weight allergens (some of which had already been defined) but the responses varied with time and between individual animals. Variability was an inherent characteristic in this study and was apparent within all strains tested except August rats. The latter were the exception, and responded in a uniform manner.

There are numerous reports of quantitative differences in the PCA titres of serum from rats of the same strain and infected at the same time with the same parasite burden (Bradbury *et al.*, 1974; Jarrett *et al.*, 1976). However, most authors accept that these differences occur but cannot provide any rational explanation for them. The Western blotting technique permits a sensitive qualitative assessment of the specific IgE response to infection. Qualitative differences in the IgE response of rats of the same strain is a relatively new finding. However, similar observations were made with Sprague-Dawley rats infected with *N. brasiliensis* when sera were tested for IgE by Western blotting (Yamada *et al.*, 1991). Kennedy *et al* (1990) proposed two explanations for this phenomenon which is also apparent in the IgG response. First, parasite burdens may vary from rat to rat, and secondly, the rats were not true inbred strains. Future research should be conducted using fully inbred strains of defined RT1 haplotype, and include a detailed study of this phenomenon and perhaps why August rats lacked demonstrable variability in the IgE response. The factors contributing to this phenomenon should also be defined.

Although variability within strains confuses the classification, F344 rats which fail to respond with specific IgE after primary infection, but which do respond after subsequent reinfection, may be classified as late responders. In contrast, LOU and Wistar strains responded as early as day 7 of infection and may,

therefore, be classified as early responders. August and Hooded Lister strains fall between these two categories. In addition, LOU rats can be classified as high responders due to the intensity of their primary response.

During helminthiasis, parasite-specific IgE accounts for only a small proportion of the total serum IgE. Similarly, the relative concentrations of specific IgG are often greater than those of IgE (Chapter 3). For this reason, detection of specific IgE within serum samples may be complicated. Kemeny and Lessof, (1987) used the radioallergosorbent and I^{125}-radiolabelled antigen binding assays to demonstrate that, for some allergens, the ratio of IgG to IgE antibody was 220:1. Similar observations were made with *N. brasiliensis* infection in mice. Pfeiffer *et al* (1983) showed that high IgE responder strains produced the greatest amounts of parasite- specific IgG_1 and, in comparison, medium and low IgE response phenotypes, produced significantly less, or no IgG_1, respectively. Generally, the allergens demonstrable in this study are also the principal antigens recognized by non-IgE isotypes (see Chapters 3 and 4). This explains why there is a strong similarity between the *N. brasiliensis* antigens defined by immunoprecipitation with immune rat IgG (Kennedy *et al.*, 1990b) and the allergens defined in Chapters 3 and 4. Kennedy *et al* (1990b) detected major antigens with MW of 12,000, 16,000, 18,000, 25,000, 29,000, 50,000, 55,000, 60,000 and 98,000 which are not dissimilar to the allergens of 14,000, 17,000, 25,000, 32,000, 60,000 and 75,000 noted in the present study.

Competitive interactions between IgE and IgG antibodies have already been noted with nematode antigens and large errors in IgE detection were reported for ELISA and RIA techniques (Hamilton, Hussain, Ottesen and Adkinson 1981). The authors concluded that IgE and IgG isotypes (IgG_1 in particular) recognized the same or overlapping epitopes. Similar observations were made in Chapter 3 in this thesis. Western blots using an IgG_1-specific probe demonstrated that this isotype bound to antigens with molecular weights similar to those noted for low molecular weight allergens. Comparable results have been reported for filariasis (Hussain and Ottesen, 1986) and schistosomiasis (Hagan, Blumenthal, Dunn, Simpson and Wilkins, 1991).

In order to compare the specificities of IgG and IgE isotypes and to improve the detection of *N. brasiliensis* allergens, IgE was purified from HIS. Isolated IgE was shown to be pure by SDS-PAGE and to be biologically active by PCA and, on Western blots, purified immune IgE recognized the major low molecular weight allergens already detected by HIS. However, due to non-specific interactions with high MW proteins, purified IgE was of limited use as a blotting reagent. The reason for these non-specific interactions requires further analysis. IgE is very labile but since purified IgE was active by PCA, it is unlikely that the purification technique was wholly responsible for non-specificity on blots. More work is, however, required to improve the blotting technique, and attempts to determine specificity by immunoprecipitation would, if successful, be very informative.

Finally, it was apparent from analysis of Western blots that non IgE-isotypes, in IgE-depleted HIS, blocked the binding of IgE to allergens in a sequential blotting procedure. It is not clear whether identical epitopes are seen by the different isotypes or whether there is steric hindrance as a result of the initial interaction of IgG with blotted allergens. This question could only readily be resolved after isolation, characterization, and amino acid sequencing of the relevant allergen and preparation of over-lapping peptides for epitope mapping. Such an approach is probably more feasible for *Ascaris* spp. where the relatively abundant allergens from *A. lumbricoides* and *A. suum* (Christie, Dunbar, Davidson and Kennedy, 1990) are immunologically similar, have similar amino acid composition, and share N-terminal amino acid sequence identity over the first 41 residues. However, the fact that blotting with HIS, in which parasite-specific IgG is abundant, still permitted the detection of *Nippostrongylus* allergens with IgE probes suggests that each isotype may recognize different epitopes or that IgE has a higher affinity for a given epitope than IgG.

Rapid expulsion (RE) of a challenge infection with *T. spiralis* by primed rats results in the elimination of greater than 95% of the infective larvae within minutes of entering the gut lumen (McCoy, 1940; Bell, McGregor and Despommier, 1979; Russell and Castro, 1979). The RE of *N. brasiliensis* is associated with activation of mucosal mast cells (Miller, Huntley, Newlands and Woodbury, 1983), and a failure of the parasite to attain its natural predilection site close to or within the gastric mucosa

(Miller, Huntley and Wallace, 1981). The rapidity of RE, therefore, together with the evidence of mast cell activation through the systemic secretion of RMCP II (Miller, Woodbury, Huntley and Newlands, 1983) suggests that IgE-mediated immediate type hypersensitivity responses are involved in worm elimination (Miller, 1984).

However, until recently, there was only indirect evidence supporting this theory. Ahmad, Hua, Wang and Bell (1991) showed that IgE, affinity purified from HIS, when given to rats adoptively immunized with primed T cells, caused the RE of a challenge infection. They showed that IgE was involved in RE but was not the sole isotype capable of inducing RE.

In the naive rat, *N. brasiliensis* and *T. spiralis* survive as adults for only a few days before they are expelled from the intestine (Jarrett *et al.*, 1968) by a gradual process known as self-cure. There is a temporal association between enhanced IgE synthesis, mastocytosis, mast cell activity and the self-cure response (Miller, 1984) although there is relatively little firm evidence to suggest IgE involvement in this response. However, it is evident from Chapters 3 and 4 and other studies (discussed in Chapter 4) that parasite-specific IgE is detectable before the commencement of parasite expulsion and therefore may still have some role in the intestinal response noted during self-cure.

Detection of IgE-bearing cells by flow cytometry in *N. brasiliensis* infection, is consistent with the increase in serum IgE levels described in kinetic studies (Jarrett *et al.*, 1976).

The same workers reported that, before parasite expulsion, serum IgE was exclusively non-specific, however it is now clear that this may not be the case and that, in some strains of rat, parasite-specific IgE is demonstrable before expulsion. A large proportion of the IgE-bearing cells carry cytophilic IgE (Urban *et al.*, 1984) and it is therefore likely that a small proportion of this cytophilic IgE is parasite-specific.

IgE-bearing cells were first detected in BM on day 10 of infection and were predominantly granulocytes and mononuclear cells. Although the BM is the primary source for tissue and blood leukocytes it is unlikely that it supplies all the IgE-bearing cells subsequently detected in PBL and in peritoneal lavage. The proportions of IgE-bearing cells in PBL and BM declined after day 15, but remained high in peritoneal lavage. Classically, IgE is associated with mast cells and basophils through Fc RI but it is associated with many other cell types through the lower affinity Fc RII. Eosinophils, macrophage/monocytes and platelets will all express IgE receptors and all can induce damage against tissue stages of some helminths (Capron *et al.*, 1985; Joseph *et al.*, 1986; Pancre *et al.*, 1988; Meleiwica and Speigelberg, 1980; Verwaerde *et al.*, 1987). However *N. brasiliensis* is a lumen-dwelling intestinal parasite that achieves its predilection site early, by day 3 of in infection, before IgE-bearing cells were noted in BM or PBL. This latter finding may reflect the absence of IgE or, alternatively, lack of activation of potential Fc_{ϵ}RII cell populations.

In contrast, on reinfection, serum titres of parasite-specific IgE rise quickly (Jarrett *et al.*, 1976) and although not measured here, IgE-bearing cells would presumably respond with similar kinetics. Therefore, in the immune animal it is not unrealistic to expect contact between IgE-bearing cells in the tissues and migrating *N. brasiliensis* larvae. Verwaerde *et al.*, 1987 have already shown that a monoclonal IgE, specific for *S. mansoni*, successfully mediated protection in naive hosts and macrophage killing of parasites *in vitro*. There is little evidence to suggest that the migrating larvae of *N. brasiliensis* are targets of immunity during secondary or tertiary infection (reviewed in Miller, 1984) but, when exposed to repeated, low dose challenge under field conditions the recruitment of Fc RII cells may prove more efficacious. Their role in the gut has yet to be explored.

The low affinity receptor, $Fc_{\varepsilon}RII$ also known as CD23 in man has been the subject of considerable interest. The proportions of CD23-positive lymphocytes are raised in atopic dermatitis (Sakamoto, Takigawa, Tamamori, Horiguchi and Yamada, 1990), rheumatoid synovitis (Hellen, Rowlands, Hansel, Kitas and Crocker, 1990) and psoriasis (Miller, Rocken, Joel, Bonnefoy, Saurat and Hauser, 1990). Cytokines such as IL-2 and particularly, IL 4 (Kawabe, Mackawa, Maeda, Hosoda and Yodoi, 1990) can induce raised expresson of CD23. It is likely that these cytokines are similarly responsible for the generalized increase in IgE-bearing cells noted during infection with *N. brasiliensis*. Recent studies on the production of IL-4 in the mouse would tend to support this conclusion.

An intriguing question, which remains unanswered, is why should mucosal mast cells apparently retain IgE within the cytoplasm, whereas connective tissue mast cells carry IgE exclusively on the surface membrane (Mayrhofer *et al.*, 1976)? The possiblity that the protease content of each mast cell subset might be responsible for these differences was considered. Our ability to purify RMCP I and II, and IgE, allowed a comparison of the catabolic activities of these enzymes against rat IgE. To further the comparison between the proteolytic activities of RMCP I and II, IgG_{2a} was included in the study. Although both enzymes cleaved IgE and IgG_{2a} isotypes, RMCP II acted more rapidly with an almost immediate depletion of Epsilon chain. Digestion of IgE resulted in immunoreactive peptides of low molecular weight (19,000 and 23,000 MW respectively) which were still demonstrable after 24 hours digestion. Proteolysis of human IgE also results in one cleavage product (40,000 MW) which is resistant to further digestion (Kolmannskog, 1985, 1986, 1987). The latter analysis was under non-reducing conditions and it is possible that the 40,000 MW product may be composed of two 20,000 MW proteins similar in size to those noted here. From the present study it was clear that RMCP I and II generated distinct cleavage products and therefore expressed distinct specificities. This confirms previous work which has addressed the specificity of the rat mast cell proteases (Woodbury *et al.*, 1989; Miller *et al.*, 1986). Furthermore, RMCP I and II had very different rates of proteolysis. Digestion of epsilon heavy chain by RMCP I was approximately 80% complete within 30 seconds but there was very little change in the staining intensity of this band thereafter. This result may indicate

heterogeneity in the IgE isotype and a variation in susceptibility to proteolysis. Lehrer *et al* (1981) noted differences in the binding capacities of several murine myeloma mouse IgE proteins to homologous mast cells which suggests that heterogeneity of the IgE isotype exists and may explain the presence of residual, protease resistant, epsilon heavy chain. Overall, however, these experiments do not resolve the apparently distinct capacities of MMC and CTMC to internalize and store intact IgE or immunoreactive IgE peptides.

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